

ANTI-CARIES EFFICACY OF FLUORIDE AT INCREASING MATURATION OF A
MICROCOSM BIOFILM

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DEDICATION

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ANTI-CARIES EFFICACY OF FLUORIDE AT INCREASING MATURATION OF A MICROCOSM BIOFILM

Dental biofilm is a main contributing factor in the initiation and progression of dental caries. The maturation of dental biofilms is expected to alter the anti-caries efficacy of fluoride compounds. In the first aim, we conducted a series of model-development experiments to test different variables to standardize a reproducible in-vitro microbial caries model. We evaluated: surface conditioning using saliva; sucrose concentrations and caries lesion severity; growth media conditions and mineral saturation; dental substrate types; pH cycling protocol characteristics. In the second aim, we used the developed model to evaluate the changes in the anti-caries efficacy of three fluoride compounds (Sodium fluoride (NaF); Stannous fluoride (SnF₂); Amine fluoride (AmF); and deionized water (DIW- negative control)) at increasing maturation of a microcosm biofilm. We continued the pH cycling protocol for 4 days, 8 days, and 12 days. We tested biofilm cariogenicity and carious lesion severity at each maturation stage. In the third aim, we used the developed model to test the effect of different exposure periods (early vs. late exposure) of the biofilm to three fluoride compounds (NaF, SnF₂, AmF, DIW) in comparison to DIW. We also evaluated the recovery of biofilm cariogenicity with each exposure period. We evaluated, for each exposure period and recovery stage, biofilm cariogenicity and carious lesion severity. We analyzed the relationships between different variables (biofilm age, fluoride compound type, exposure period) using ANOVA models. In conclusion: 1. The present model allows testing the effect of biofilm maturation on the anti-caries efficacy of fluoride compounds. 2. Biofilm

maturation plays an important role in increasing biofilm tolerance against fluoride treatment; it could also influence the selection of fluoride compounds to achieve optimum cariostatic effect. 3. Exposure period, and type of fluoride compound, both influence the biofilm tolerance to fluoride anti-caries effect; they may also result in a sustainable release of fluoride over time.

Frank Lippert, PhD, Chair

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CHAPTER 1: INTRODUCTION

1.1. Introduction

Dental caries is a multifactorial disease, where acid-producing bacteria, dietary carbohydrates, time, and a susceptible host are all factors contributing to the initiation and progression of the disease.^{1, 2} The oral cavity harbors over 700 bacterial species.³ Dental biofilm has been defined as "matrix-enclosed microbial communities in which cells adhere to each other and/or to surfaces or interfaces".⁴ Dental biofilm formation consists of several steps: formation of acquired pellicle; initial adhesion; subsequent maturation; and dispersion of biofilm.⁵ The biofilm produces, and encloses itself in, extracellular polymeric substance (EPS) that serves many functions.⁵

Oral bacteria are usually present in the oral cavity in an equilibrium state. In the presence of carbohydrates in the oral environment, bacteria shift to an increased population of acidogenic (acid-producing) and aciduric (acid-tolerant) bacteria.¹ The biofilm bacteria start metabolizing these carbohydrates using biochemical processes such as the glycolytic pathway, where glucose is fermented to pyruvate, which metabolizes to lactic acid as an end product.^{6, 7} The presence of acids over dental structures leads to the initiation of carious lesions through disrupting the mineral equilibrium of dental structures (i.e. enamel and/or dentin).^{1, 8}

Targeting the biofilm as a contributing factor to dental caries is an approach taken to control the disease.^{6, 7} Mechanical removal of dental biofilm is an effective way to break the caries process. However, biofilm starts to re-form immediately afterwards. Therefore, antimicrobial/anticaries agents are used to target the biofilm virulence factors. Some of these virulence factors are acid production (acidogenicity) and

glucosyltransferases (Gtfs) and EPS formation.^{7, 9, 10} Fluoride is well known as the gold standard anticaries agent.¹¹ Fluoride in toothpastes, can be in the form of sodium fluoride (NaF), sodium monofluorophosphate (MFP), stannous fluoride (SnF₂), and amine fluoride (AmF).¹² In addition to its effect on enhancing remineralization and preventing demineralization of tooth structures, fluoride in its various compounds has the potential to inhibit bacterial virulence factors.¹³

Biofilm maturation involves changes in the composition and architecture of the biofilm.¹⁴ These changes are expected to modulate the interaction between the biofilm and surrounding environment (which includes antibacterial/anticaries agents). For example, biofilm may serve as a diffusion barrier against anticaries agents from reaching dental surfaces.¹⁵ Therefore, testing the interaction between the biofilm and these agents at each maturation stage is necessary to obtain an optimum cariostatic level against oral bacteria.^{15, 16}

Another critical area to be studied is the immediate and long-term recovery of dental biofilm after being exposed to fluoride treatments. Some previous studies suggested that the brief fluoride treatment obtained through oral hygiene measures may allow the biofilm to recover after a certain time,^{6, 10, 17} especially that the main goal is affecting biofilm's virulence and not viability.^{6, 7, 17-19}

To study these areas of research, there is an increased need to develop clinically relevant in-vitro microbial caries models. The existing well-established models that are widely used in in-vitro studies still do not incorporate the microbial component as part of the caries process.^{20, 21} Developing such a model is still challenging because of the complexity of the oral environment and the diversity of oral bacteria.²² Previous studies

that tested the interaction between dental biofilm and antimicrobial agents have focused on more simple, controllable conditions. For example, they tested the biofilm at a certain age, or at two time points.^{1, 2, 23, 24}, or used single- or two-species biofilms.^{1, 23, 24}

Some of the characteristics to be tested as components of an ideal model may include: biofilm's composition (i.e. single- vs multi-species biofilm); dental substrate type (human vs. bovine); growth medium conditions (i.e. mineral contents and clinical relevance-pH challenges); and hard tissue status (i.e. sound enamel or [biofilm-induced] lesion).

1.2. Project Aims

This dissertation presents four specific aims, individually described in chapters:

Specific Aim 1 (Chapter 2): To explore the influence of salivary conditioning prior to biofilm formation on enamel demineralization.

Specific Aim 2 (Chapter 3): First, to evaluate the use of human and bovine enamel specimens in microbial caries models. Second, to test the concept of creating niches over the substrate surface to enhance the biofilm's adhesion to the surface. Third, to test all of these variables at different biofilm maturation stages.

Specific Aim 3 (Chapter 4): To explore the anti-caries efficacy (cariou lesion severity and biofilm cariogenicity) of three fluoride compounds, NaF, SnF₂, AmF, at increasing maturation stages of a microcosm biofilm grown on bovine enamel.

Specific Aim 4 (Chapter 5): First, to explore the influence of biofilm maturation and time of exposure to fluoride treatments on fluoride anticaries efficacy (cariou lesion

severity and biofilm cariogenicity). Second, to explore the recovery of biofilm cariogenicity after exposure to fluoride treatments using two exposure periods.

CHAPTER 2: THE INFLUENCE OF SALIVARY CONDITIONING ON BIOFILM-MEDIATED ENAMEL DEMINERALIZATION- A PILOT STUDY

2.1. Introduction

Dental caries is a multifactorial disease, where acid-producing bacteria, dietary carbohydrates, time, and a susceptible host are all factors contributing to the initiation and progression of the disease.¹ The process starts when oral bacteria, which are present in an equilibrium state, ferment carbohydrates; this equilibrium shifts to increased populations of acidogenic (acid-producing) and aciduric (acid-tolerant) bacteria.¹ The consistent presence of acid in the environment causes disruption in the mineral equilibrium of the exposed dental structures (i.e. enamel and/or dentin), and therefore, leading to the initiation of carious lesions.^{1, 8}

Dental biofilm has been defined as “matrix-enclosed microbial communities in which cells adhere to each other and/or to surfaces or interfaces”.²⁵ Over 700 bacterial species are present in the oral cavity.³ They are present on all oral hard and soft tissue structures. These bacterial aggregations usually produce, and become enclosed in, extracellular polymeric substance (EPS). The formation of dental biofilm (or dental plaque) consists of several steps, which start with the formation of the acquired pellicle, followed by the initial adhesion of planktonic bacteria to the pellicle layer through binding sites, subsequent maturation of the bacterial biofilm, and, finally, the dispersion of biofilm through detachment of cells/clusters of cells.⁵

The formation of the acquired pellicle is the first step in dental biofilm formation, and it is a unique step distinguishing it from other biofilm types.⁵ It consists of several interactions between various salivary glycoproteins, and their interaction with the tooth

surface. These biochemical interactions are based on Gibbs law of free enthalpy^{5, 26}; they lead to the attachment of salivary glycoproteins to a surface (i.e. the enamel). The resulting formed layer is a protein-rich layer with binding sites; these are the sites ready for the attachment of early colonizers.²⁶

Based on this unique process, some studies suggested a new intervention to prevent biofilm formation: this intervention is in the form of preventing pellicle formation.²⁷ Many microbial studies have explored and studied the dental biofilm from many aspects, and using different cariogenic models.^{7, 19, 23, 28-32} However, they omitted the step of surface conditioning through the formation of acquired pellicle. This leads to less clinical relevance especially that this area of study (the significance of including the pellicle) has not been researched previously.

Acquired enamel pellicle (AEP) has been explored previously for its composition and function.³³⁻³⁷ Studies have explored pellicles and found differences between AEP formed in-vitro, in-vivo, and also in-situ. The variations include ultrastructural variations,³³⁻³⁷ intrinsic and extrinsic maturation variations, as well as the morphology of the AEP. Studies have found that in-vitro AEP were superior to in-vivo in which they contain higher amounts of proteins. They are also superior in the amounts produced (due to the difficulty in collecting in-vivo AEP).³³⁻³⁷

In in-vitro studies, the salivary pellicle is typically allowed to form before exposure to bacteria-containing media, resulting in biofilm formation. Several methods for the formation of a salivary pellicle have been utilized.³⁸⁻⁴⁰ In general, the dental surface is exposed to saliva (sterilized, free from bacteria) for a specific amount of time (ranges from minutes to several hours) before being exposed to oral bacteria for biofilm

formation.³⁸⁻⁴⁰ The significance of surface conditioning before biofilm growth (to allow the formation of acquired enamel pellicle) in studying biofilm models was not evaluated previously and, therefore, needs to be explored. Therefore, the aim of this study was to explore the influence of salivary conditioning prior to biofilm formation on enamel demineralization.

The null hypotheses for this research were: 1) There is no significant difference between pasteurized saliva, filtered non-sterilized saliva, and de-ionized water (DIW; negative control) as conditioning agents on biofilm-mediated enamel demineralization; 2) There is no significant difference between 0.5% and 1% sucrose-supplemented growth media on enamel demineralization.

2.2. Materials and Methods

Specimen Preparation

Extracted bovine incisors were sectioned to obtain 5×5 mm enamel specimens using a Buehler IsometTM low-speed saw (Buehler, Ltd., Lake Bluff, IL, USA). Approximately 54 teeth were used to obtain 54 specimens. During preparation, the teeth were stored in deionized water with thymol. Using a Struers Rotopol 31/Rotoforce 4 polishing unit (Struers Inc., Cleveland, PA, USA), all specimens were ground and polished to ensure flat parallel dentin/enamel surfaces. For the finishing process, the dentin side was ground using 500-grit silicon carbide grinding paper. Then, the enamel side was serially ground using 1,200, 2,400 and 4,000 grit papers. After that, polishing of the specimens took place using 1 μ m diamond polishing suspension on a polishing cloth to obtain a 5×5 mm polished enamel surface. All specimens were checked for cracks,

white spots, or any other flaws that could lead to excluding the specimen from the study, using Nikon SMZ 1500 stereomicroscope at $\times 20$ magnification.

Baseline Measurement and Experimental Groups

All specimens were subjected to enamel surface microhardness testing (VHN_{sound}) to ensure standardization. A Vickers diamond identifier (Tukon 2100; Wilson-Instron, Norwood, MA, USA) was used with a load of 200 g for 15 s. Three indentations, approximately 100 μm apart, were placed on each specimen and averaged; the inclusion range was VHN_{sound} between 300-380. Specimens were divided into two groups, based on the sucrose concentration the biofilm/enamel surface was subjected to (0.5% and 1% sucrose concentrations). Each group was divided further into 3 subgroups ($n=9/\text{subgroup}$), based on the nature of the salivary conditioning to the enamel surface prior to biofilm formation. The three conditions tested were: pasteurized saliva; filtered, non-sterilized saliva; and de-ionized water (DIW; negative control).

Salivary Bacterial Model

Biofilm Model

After specimen preparation was completed, specimens were mounted on the inside of a lid of a 6-well plate (FisherBrand, Fisher Scientific), with three specimens per well, using acrylic cubes to create an active attachment model, following a previously described protocol.^{1, 23} The model was disinfected using 70% ethanol prior to bacterial and/or pellicle inoculation.⁴¹

Saliva Collection

Ethical approval was obtained from the IUPUI institutional review board (IRB #1406440799) for saliva collection. Wax-stimulated saliva samples from three donors were collected and pooled (approx. 50 ml/donor). The inclusion criterion included: healthy participants (no systemic diseases) with normal salivary flow and no presence of active caries or periodontal disease. To ensure standardization, participants refrained from oral hygiene measures overnight. Prior to bacterial inoculation or freezing, the pooled saliva was tested for the presence of *Streptococcus mutans* and *Lactobacilli* using selective agars (MSSB and Rogosa agars, respectively). The results confirmed the presence of both species. Five ml of the pooled saliva and growth media mix (1:10 ratio) were incubated overnight, then mixed with 10% glycerol and frozen immediately at -80° C, this microcosm bacterial mix was used as the source for bacterial inoculum. The remaining pooled saliva was pasteurized as described below.

Saliva Pasteurization

The collected, pooled saliva was diluted in sterile saline at 1:10 dilution. The diluted solution was filtered using Watman filter paper to remove large debris. This filtered saliva was used to create the salivary pellicle in subgroups exposed to filtered, non-sterilized saliva. For pasteurization, the remaining filtered saliva was subject to an additional sterilization step, pasteurization, using a previously published protocol.⁴²

Briefly, after the diluted solution was first filtered, it was centrifuged to remove mucin and bacteria (10 minutes, 4°C, 27,000 x g). The supernatant was retained and

pasteurized at 60°C for 30 minutes, then re-centrifuged for 10 minutes. The prepared saliva was stored in aliquots of 50 ml and frozen at -80°C for further use.

Surface Conditioning

All specimens were immersed in their corresponding solutions: pasteurized, wax stimulated saliva; wax-stimulated, filtered, non-sterilized saliva; DIW as negative control. Specimens were incubated in their respective solution at 5% CO₂ and 37°C for 5 minutes to allow surface conditioning.

Biofilm Growth

Immediately after surface conditioning, specimens were transferred to a new, sterile 6-well plate containing growth culture media that was inoculated with the overnight bacterial culture (without washing the samples between the two steps).

Microcosm biofilm was grown under anaerobic conditions at 5% CO₂ and 37° C for 48 hours. The growth media used to grow the biofilm was Brain Heart Infusion (BHI) broth, supplemented with 5 g/l yeast extract and 5% vitamin K and hemin (v/v) and supplemented with either 0.5% sucrose or 0.1% sucrose. After 48 hours, the biofilm was collected through placing each specimen in an Eppendorf tube (containing 1 ml sterile saline), sonicated at 30W for 10 seconds, then vortexed immediately for 10 seconds for complete biofilm detachment from the enamel surface.

Post-treatment Analyses

Surface Microhardness Change (VHN_{change})

Post-treatment surface microhardness was measured following the same protocol used for the VHN_{sound} . Three indentations were made at approximately 100 μm next to the baseline VHN_{sound} indentations. The VHN_{change} values were calculated using the formula $VHN_{change} = 100 * (VHN_{sound} - VHN_{post}) / VHN_{sound}$.

Transverse Microradiography

One section, approx. 100 μm thick, was cut from the center of each specimen and across the specimen using a Silverstone-Taylor Hard Tissue Microtome (Scientific Fabrications Laboratories, USA). All sections were placed in the TMR-D1 v.5.0.0.1 system and X-rayed at 45 kV and 45 mA at a fixed distance for 12 s. An aluminum step wedge was X-rayed under identical conditions. Digital images were analyzed using the TMR software v.3.0.0.18. Sound enamel was assumed to be 87% v/v mineral. The data obtained from this analysis were integrated mineral loss (ΔZ) and lesion depth (L).

Statistical Analysis

All three variables (VHN_{change} , ΔZ , L) were analyzed using two-way ANOVA, where sucrose concentration and surface conditioning were the factors analyzed individually as well as the interaction between them. All pair-wise comparisons from ANOVA analysis were made using Fisher's Protected Least Significant Differences to control the overall significance level at 5%.

2.3. Results

The two-way interaction sucrose concentration \times surface conditioning was not significant for VHN_{change} ($p=0.872$), ΔZ ($p=0.662$) or L ($p=0.436$). Surface conditioning affected VHN_{change} significantly ($p=0.0079$); however, it did not affect ΔZ ($p=0.7383$) or L ($p=0.7323$). Sucrose concentration did impact ΔZ ($p<0.0001$) and L ($p<0.0001$); however, it did not affect ΔVHN ($p=0.2877$). For better clarity, Table 2.1 shows the data for all measured variables for each subgroup.

The pairwise multiple comparison analyses of the VHN_{change} indicated that the pellicle type created a significant difference between groups. In both sucrose concentrations, surface conditioning with pasteurized saliva resulted in the lowest VHN_{change} values, when compared to other surface conditioning groups. The difference between pasteurized subgroups and the two other surface conditionings was significant (pasteurized and filtered saliva subgroups $p=0.006$; pasteurized and DIW subgroups $p=0.0075$), while there was no significant difference between filtered saliva and DIW subgroups ($p=0.9312$) (Table 2.1).

For the ΔZ values, the pairwise comparisons indicated a statistically significant difference only between 0.5% and 1% sucrose concentration ($p<0.0001$), and not based on the surface conditioning status. Growing the biofilm in 1% sucrose always resulted in lesions with higher ΔZ values, indicating more severe lesions.

Similarly, the pairwise comparisons for L values indicated a statistically significant difference between 0.5% and 1% sucrose ($p<0.0001$). Also, the L values were always higher in 1% incubation conditions, which means more severe carious lesions (Table 2.1).

2.4. Discussion

In this study, we aimed to evaluate the influence of surface conditioning using human saliva prior to biofilm formation in-vitro on enamel demineralization. The results of the statistical analysis showed that the hardness data were only affected by pellicle type, whereas the TMR data were only affected by sucrose concentration. To fully understand this contradiction, we need to consider the differences between the studied variables.

Surface microhardness is a measurement of how a material responds to deformation. It is mainly influenced by surface integrity and not necessarily by structural characteristics or mineral content of the bulk substrate. One of the functions of the pellicle in the oral cavity is its masking effect: it coats dental surfaces, and other structures, and this may lead to different patterns of bacterial biofilm formation according to the presence/absence or the quality of the pellicle.⁴³⁻⁴⁵ The presence or absence of a pellicle layer, therefore, will undoubtedly affect surface characteristics, and this may explain the significant differences among pellicle subgroups in our study. On the other hand, TMR measures are based on mineral content rather than structure. Therefore, we expect to observe differences only when carious lesions with different mineral contents and/or distributions form during demineralization.⁴⁶

Surface microhardness testing is straightforward and nondestructive. It is coupled with transverse microradiography in some studies based on the objective of the study. The loss of minerals within the outer enamel was found to be proportional with the degree of the indenter's penetration. However, deeper lesions cannot be quantitatively measured using surface microhardness.⁴⁷ Moreover, surface microhardness is most

effective in analyzing homogenous materials and shallow lesions only (e.g. enamel outer surface).²¹ White²¹ (1987) reported in a study, where they evaluated the differences between surface microhardness and microradiography, that surface microhardness was able to detect remineralization in early lesions (or at least hardening of the surface without remineralization).²¹ It is difficult to evaluate mineral content within the outermost layers of the enamel using microradiography. Therefore, the two analyses are usually considered complementary to each other in demineralization/remineralization studies.²¹

In this study, we used an active attachment model which was adopted from a previously published model.^{1, 23} Although this model still lacks more complex features that lead to more clinical relevance (e.g. pulsation of nutrients into the environment),² an active attachment has the advantage of ensuring that the bacterial layers formed over the surface are not just sedimented cells, but rather attached to the enamel surface and to each other.²³

We used a salivary bacterial mix to create a largely undefined microcosm biofilm. Prior to bacterial inoculation, the pooled saliva was tested for the presence of *Streptococcus mutans* and *Lactobacilli* using selective agars. The results confirmed the presence of both species. In-vitro studies utilize a variety of approaches with biofilms formed from monospecies (such as *Streptococcus mutans* or *Lactobacilli*),^{23, 24} two or multiple species (3-10 species),^{1, 48} or a microcosm biofilm.^{49, 50} While single or multiple, defined species allow for greater control, employing a microcosm biofilm can result in greater clinical relevance. The acquired pellicle can be formed from saliva or plaque samples collected and pooled from single and/or multiple donors.⁴¹ We chose the present approach based on conclusions drawn from previous studies.^{41, 49} Some studies limited

their salivary (or plaque) mix to be collected from a single donor,⁵⁰ other studies collected samples from two or more donors.⁵¹ In our study, we collected wax-stimulated saliva samples from three donors and pooled them, thereby increasing the translational value of the present findings.

One could suggest that using a microcosm biofilm source may result in large variability. The variability of biofilm characteristics in in-vitro studies was explored previously.⁴¹ Most studies⁵⁰⁻⁵² concluded that collection of saliva samples from the same donor at different times did not affect biofilm diversity. Moreover, the involvement of sucrose over time can lead to a dominance of certain bacterial strains (mainly cariogenic bacteria), thus overcoming initial differences between different samples (either from different donors or collected at different times from the same donor).⁴¹

The formation of acquired pellicle in in-vitro studies can be conducted through exposing the surface of interest to sterile saliva solution for a certain period of time. Although including salivary pellicle in the model seems to give more clinical relevance, this step requires an expensive and time-consuming saliva sterilization (to ensure a bacteria-free solution that still contains salivary glycoproteins).

It was documented previously that the time required for the formation of the pellicle ranges from 3 minutes to 7 days. The same studies reported minor relevance of pellicle's maturation (i.e. aging).⁵³⁻⁵⁵ Based on that, we chose to incubate, in this study, the enamel samples in three surface conditioning media types for five minutes.

The second variable we explored in this study is sucrose concentration. Carbohydrate concentration within the growth media has been reported to have an impact in the biofilm composition.^{56, 57} Consequently, we assume that the cariogenicity of the

biofilm may also be affected. As mentioned earlier, acquired pellicle formation is an integral step that precedes bacterial attachment to dental and oral surfaces. The formation of acquired pellicle generally consists of two stages.⁵⁸ The first stage is very rapid and includes adsorption of salivary glycoproteins to the substrate. However, the second stage occurs immediately after the first stage *in vivo*.⁵⁸ It is characterized by more adsorption of biomolecules, where the oral fluids are the source of these biomolecules.⁵⁸ Therefore, we included in our study two different types of sources for the salivary pellicle to represent these two stages and explore their influence in the pattern of demineralization. Although salivary pellicle that forms from pasteurized saliva (which becomes bacteria-free) makes the *in-vitro* study more controllable and the model more applicable if used in studies involving single/multiple species biofilm, using filtered/non-pasteurized saliva ensures more clinical relevance as the only eliminated element is food debris.

Our study focused mainly on the pellicle involvement in *in-vitro* microbial studies, and the influence of this factor on the hard tissue substrate characteristics. This study did not test the influence of the presence of acquired pellicle on the cariogenicity of a microcosm biofilm. This can be tested in a similar study thorough collecting 48-hour biofilm and analyze its cariogenicity (e.g. lactic acid production). Another factor that may be tested is the bacterial source (i.e. saliva vs. plaque samples), since it was already reported that biofilms formed from a saliva versus plaque as sources have different characteristics.⁴¹ Furthermore, different incubation times may affect pellicle formation and maturation. Lastly, pellicle formation can also be achieved by exposing specimens to the oral cavity for different periods of time, which provides another avenue for future research.

2.5. Conclusion

Bearing in mind the limitations of the present study, the presence or absence of an artificially induced acquired pellicle layer does not influence biofilm-mediated enamel caries lesion formation as measured by TMR. Some differences were observed using surface microhardness, indicating a complex interaction between pellicle proteins and biofilm-mediated demineralization of the enamel surface.

Table 2.1. Carious lesion severity- percentage surface microhardness (VHN_{change}), mineral loss (ΔZ ; %volmin $\times\mu\text{m}$), and lesion depth (L; μm). Average values in enamel samples subject to either 0.5% or 1% sucrose solutions, and to different types of surface conditioning

Sucrose Conc.	Surface Conditioning Mean (SD)																	
	Pasteurized						Filtered, non-sterilized						Control (DIW)					
	VHN _{change}		ΔZ		L		VHN _{change}		ΔZ		L		VHN _{change}		ΔZ		L	
0.50%	32.7 (20.7)	a	820 (266)	A	38.8 (9)	A	51.5 (18.4)	b	1257 (406)	A	47.8 (9.6)	A	48 (17.1)	b	867 (338)	A	37.3 (14.2)	A
1%	24.8 (22.4)	a	2623 (1014)	B	78.4 (17.5)	B	43.9 (21)	b	2505 (1480)	B	73.3 (26.7)	B	46.2 (18.8)	b	2428 (1208)	B	74.6 (20.6)	B
Lower case: significance between surface conditioning status																		
Upper case: significance between sucrose concentrations																		

CHAPTER 3: COMPARISON OF HUMAN AND BOVINE ENAMEL IN A MICROBIAL CARIES MODEL AT DIFFERENT BIOFILM MATURATIONS

3.1. Introduction

Dental biofilms play a major role in caries lesion development through fermenting carbohydrates and producing acids. Dental biofilm maturation (i.e. the biofilm age) involves changes in biofilm composition and architecture.¹⁴ Consequently, biofilm interactions with the surrounding environment, and with anticaries agents as well, are expected to alter. The complex oral environment, and the diversity of the dental biofilm makes studying these changes during biofilm maturation challenging.²² It has been documented that the biofilm could act as a diffusion barrier preventing anticaries agents from reaching the tooth surface.¹⁵ This happens because proteins and vitamins in the environment (i.e. the growth media if in-vitro) block the biofilm-enamel interface and restrict the diffusion of the ions into enamel surface.^{32, 59} However, there is a need for further research in this area for better understanding.

An approach taken in utilizing caries preventive agents is targeting the biofilm's virulence factors. For example, fluoride is used as an antibacterial agent; it affects bacterial acidogenicity, acidity, as well as exopolysaccharide (EPS) formation.⁶⁰ A wide range of model systems has been introduced to test anticaries agents, with in vitro caries models being used most frequently.

Many studies have tested the biofilm and its cariogenicity.^{9, 18, 23, 61-63} Some studies limited their methodologies to simpler approaches to achieve a reproducible, controllable model (e.g. microtiter plate, single-species biofilm); other studies aimed to maintain clinical relevance (e.g. artificial mouth models, constant-depth film

fermenters).^{9, 10, 18, 61, 63} Each model has its strengths and limitations. Some of the characteristics to be tested as components of an ideal model may include: biofilm's composition (i.e. single- vs multi-species biofilm); dental substrate type (human vs. bovine); growth medium conditions (i.e. mineral contents and clinical relevance-pH challenges); hard tissue status (i.e. sound enamel or [biofilm-induced] lesion).

Both human and bovine teeth are widely used in dental studies. Although they were tested previously for their structural differences in caries studies,⁴⁶ no prior studies have explored the difference in patterns of biofilm-induced lesions between substrate types.

Studying the interaction between fluoride compounds and dental biofilm at different maturation stages is critical to fully understand the role of the biofilm in the caries process and how, as it matures, it modifies the antibacterial/anticaries effect of fluorides. Another factor that has been reported previously as a limitation to the reliability of microbial studies is achieving an evenly grown biofilm over the substrate surface, especially when growing the biofilm for relatively long periods.^{48, 64-66} This is a challenge because the variability in thicknesses, and therefore composition, of the biofilm over the surface may result in a large variability in the lesion formed and the characteristics of the biofilm itself from an area to another within the same surface.^{48, 64-66}

Therefore, we explored in this study different variables as major components of a microbial caries model. First, we evaluated the use of human and bovine enamel specimens. Second, we tested the concept of creating niches over the substrate surface to enhance the biofilm's adhesion to the surface. Third, we tested all these variables at different biofilm maturation stages.

3.2. Materials and Methods

Study Design

Biofilm, obtained from human saliva, was grown on human and bovine enamel specimens of known Vickers hardness values (VHN_{sound}) in Brain Heart Infusion (BHI) media (with 0.2% sucrose) for 24 hours. Then, the pH cycling phase began: it included two 5-minute treatment periods, three 2-hour demineralization challenges, and four 15-minute remineralization periods. Twenty-four sub-subgroups were included in the study. The variables between sub-subgroups were based on the number of pH cycling days (4 days [4D]; 8 days [8D]; 12 days [12D]), the involvement of a protective mesh over the samples, and treatment type (18.4 mM NaF or de-ionized water (DIW)).

At the end of each pH cycling period, enamel specimens were analyzed for caries lesion severity: surface microhardness (VHN_{change}); and transverse microradiography (integrated mineral loss [ΔZ] and lesion depth [L]). The biofilm was collected and analyzed for its cariogenicity: lactic acid production (LDH activity); exopolysaccharide (EPS) amount; and viability (12 days sub-groups only).

Specimen Preparation

Extracted human and bovine incisors were sectioned to obtain 4×4 mm enamel specimens using a Buehler IsometTM low-speed saw (Buehler, Ltd., Lake Bluff, IL, USA). Approximately 108 teeth were used to obtain 216 specimens. During preparation, the teeth were stored in deionized water with thymol. Using a Struers Rotopol 31/Rotoforce 4 polishing unit (Struers Inc., Cleveland, PA, USA), all specimens were ground and polished to ensure flat parallel dentin/enamel surfaces. For the finishing

process, the dentin side was ground using 500-grit silicon carbide grinding paper. Then, the enamel side was serially ground using 1,200, 2,400 and 4,000 grit papers. After that, polishing of the specimens took place using a 1 μ m diamond polishing suspension on a polishing cloth to obtain a 4 \times 4 mm polished enamel surface. All specimens were checked for cracks, white spots, or any other flaws that could lead to excluding the specimen from the study, using a Nikon SMZ 1500 stereomicroscope at \times 20 magnification.

Baseline Measurements and Experimental Groups

All specimens were subject to enamel surface microhardness testing (VHN_{sound}) to ensure standardization. A Vickers diamond identifier (Tukon 2100; Wilson-Instron, Norwood, MA, USA) was used with a load of 200 g for 15 s. Three indentations, approximately 100 μ m apart, were created on each specimen and averaged; the inclusion range was VHN_{sound} between 300-380.

Specimens of each substrate type (i.e. human vs. bovine) were divided into three groups, based on the pH cycling duration (4D, 8D, and 12D). Each group included two subgroups (n=18/subgroup), depending on whether or not the specimens were covered with a protective mesh. Each subgroup was divided into two sub-subgroups, based on the treatment type used: 18.4 mM NaF vs. deionized water (DIW).

Specimens were mounted on the inside of a lid of a 24-well plate (FisherBrand, Fisher Scientific) using acrylic cubes to create an active attachment model, following a previously described protocol.²³ For the "mesh-covered" subgroups, specimens were covered with utility mesh-like fabric that is composed of 70% Poly Vinyl Chloride

(JoAnn Fabrics, item # 10173334). The model was disinfected using 70% ethanol prior to biofilm growth.⁴⁰

Salivary Bacterial Model

Saliva Collection

Ethical approval was obtained from the IUPUI institutional review board (IRB #1406440799) for saliva collection. Wax-stimulated saliva samples from three donors were collected and pooled (approx. 50 ml/donor). The inclusion criteria included: healthy participants (no systemic diseases) with normal salivary flow and no presence of active caries or periodontal disease. To ensure standardization, participants refrained from oral hygiene measures overnight. Prior to bacterial inoculation or freezing, the pooled saliva was tested for the presence of *Streptococcus mutans* and *Lactobacilli* using selective agars (MSSB and Rogosa agars, respectively). The results confirmed the presence of both species. Five ml of the pooled saliva and growth media mix (1:10 ratio) were incubated overnight, then mixed with 10% glycerol and frozen immediately at -80° C, this microcosm bacterial mix was used as the source for bacterial inoculum.

Biofilm Growth

Biofilm was allowed to grow on the enamel specimens for 24 hours at 37° C in the growth media. The growth media for this model contained Brain Heart Infusion (BHI) broth, supplemented with 5 g/l yeast extract (YE), 1 mM CaCl₂·2H₂O and 0.2% sucrose. After 24 hours, pH cycling protocol started, as shown in Figure 3.1.

pH cycling Model

After the 1-day biofilm growth on enamel specimens, the biofilm model was subject to a cariogenic pH cycling model, which was modified from the model used by Zhang et al.³² (2015). In this pH cycling model, the growth media was used as both the remineralization (remin) and demineralization (demin) solutions (Figure 3.1). Both the remin and demin media contained BHI broth, 5% YE, 1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.

Using 1 mM acetic acid, the pH of culture medium was adjusted to 7 (remin), and 4.5 (demin). The sucrose concentration also differed in remineralization (no sucrose) and demineralization (1% sucrose) solutions. Between treatments, the biofilm was washed by immersing the model in 0.9% sterile saline for 2 minutes. The pH cycling model was conducted daily, the biofilm and specimens were incubated overnight in remin media. For each subgroup, two treatment types were tested: 18.4 mM NaF and de-ionized water as a negative control (DIW).

At the end of each pH cycling period (4D, 8D, 12D), biofilm collection took place by carefully removing biofilm-covered enamel specimen using tweezers, then placing each specimen in an Eppendorf tube containing 1 ml sterile saline; sonicating at 30 W for 10 seconds, and vortexing immediately for 10 seconds for complete biofilm detachment from the enamel surface. For the mesh-covered sub-subgroups, the protective mesh was removed using a tweezer then was discarded before dislocating the specimen and placing it in the Eppendorf tube.

Post-treatment Analysis

Enamel Substrate

- Surface Microhardness Change (VHN_{change})

Post-treatment surface microhardness was measured following the same protocol used for the VHN_{sound} . The VHN_{change} values were calculated using the formula $VHN_{\text{change}} = 100 * (VHN_{\text{sound}} - VHN_{\text{post}}) / VHN_{\text{sound}}$.

- Transverse Microradiography

One section, approx. 100 μm thick, was cut from the center of each specimen and across the specimen using a Silverstone-Taylor Hard Tissue Microtome (Scientific Fabrications Laboratories, USA). All sections were placed in the TMR-D1 v.5.0.0.1 system and X-rayed at 45 kV and 45 mA at a fixed distance for 12 s. An aluminum step wedge was X-rayed under identical conditions. Digital images were analyzed using TMR software v.3.0.0.18. Sound enamel was assumed to be 87% v/v mineral. The data obtained from this analysis were integrated mineral loss (ΔZ) and lesion depth (L).

Biofilm Analysis

- Lactic Acid Production: LDH Assay

In order to determine live biofilm metabolism, lactic acid production was determined using a LDH cytotoxicity assay, following a previously published protocol.⁶⁷ For each sample, 45 μl of the collected, suspended biofilm was mixed with 5 μl of the LDH Assay Lysis Solution in 96-well microtiter plates, and incubated at 37° C for 45 minutes. Then, 100 μl of LDH Assay mixture was added to the cell lysate (LDH Assay Cofactor Preparation: LDH Assay Substrate: LDH Dye Solution = 1:1:1). The mixture

was kept in the dark and incubated at room temperature for 30 minutes. To terminate the reaction, 50 µl of the Stop Solution was added to the mixture. We measured absorbance readings of each well at OD_{490nm} and the background absorbance at OD_{690nm}. The background absorbance values were subtracted from the primary readings.

- EPS amount: Phenol-Sulfuric Acid Colometric Assay

Analyzing the EPS activity was performed using a previously described protocol.⁶⁸ Briefly, 50 µl of the biofilm of each sample was transferred to a 96-well microtiter plate. For each sample, 150 µl of concentrated sulfuric acid was added. Immediately after that, 30 µl of a 5% phenol solution was added to the mixture and heated to 90° C for 5 minutes. After cooling the plate at room temperature for 5 minutes the absorbance was measured at OD_{750nm}.⁶⁸

All LDH and EPS values were normalized using the protein concentration of each biofilm sample, determined by the use of the RC DC Protein Assay (Bio-Rad Laboratories, Inc. Hercules, CA, USA) following the manufacturer's instructions.

- Bacterial Viability (12 days old sub-groups)

At the last time point, biofilm samples from all sub-groups were serially diluted to 1:10⁻³, 1:10⁻⁴, and 1:10⁻⁵ (using 0.9% sterile saline). Samples were plated on Blood Agar Plates (Thermo Scientific™, Remel, Lenexa, KS, USA) using a Spread Plate Procedure.⁶⁹ To determine bacterial counts, mean log₁₀ CFU/ml values were calculated.

Statistical Analysis

VHN_{change}, ΔZ , L, LDH activity and EPS amount, were analyzed using four-way ANOVA, with factors for time, substrate type, mesh factor, and treatment type. Also, two-way, three-way and four-way interactions among the variables were analyzed.

For the viability data (12-day sub-groups), log₁₀ CFU/ml were analyzed using three-way ANOVA, with factors for substrate type, mesh factor, and treatment type, as well as all the two-way and three-way interactions among the variables. All pair-wise comparisons from ANOVA analysis were made using Fisher's Protected Least Significant Differences to control the overall significance level at 5%.

3.3. Results

ANOVA analyses of lesion severity showed that the three-way interaction among pH cycling time, substrate type and treatment type were significant in VHN_{change} ($p < 0.0005$), ΔZ ($p = 0.0027$), and L ($p < 0.0001$). Therefore, when testing the significance for carious lesion severity, the mesh factor was excluded.

The VHN_{change} data showed an increased severity of the lesions as pH cycling time increases, in both treatment and control groups. Moreover, the VHN_{change} data showed a treatment effect in all timepoints.

In general, applying a protective mesh resulted in having lower VHN_{change} values when compared to non-mesh groups. Finally, although most of the bovine groups showed a statistically significant difference when compared to human groups, the 12-day control groups were not significantly different (Table 3.1).

Regarding ΔZ and L data, an increased severity of the lesions was observed in longer pH cycling durations, especially in control groups. Lesions in the 12-day groups, both treatment and control, were more severe in bovine specimens; the difference was significant in controls only, for both ΔZ and L. Finally, there was an obvious treatment effect as the lesion progresses with biofilm maturation; this effect was more noticeable in bovine specimens, where there was no significant difference between 4, 8, and 12-day treatment groups (Table 3.1).

ANOVA analyses for both LDH activity and EPS amounts showed that the four-way interaction among tested variables (pH cycling time, substrate type, protective mesh, and treatment type) was significant (LDH $p=0.0100$; EPS $p<0.0001$).

Within all groups, it was observed that specimens covered with protective mesh showed lower cariogenicity (LDH and EPS data) of the biofilm, especially in 8D and 12D groups, in both treatment and control groups, and in both substrate types (Table 3.2). Both substrate types allowed the same trend of cariogenic activity of the biofilm: first, biofilm's cariogenicity increased as the biofilm matures; second, an increased treatment effect was observed with time (Table 3.2).

For biofilm's viability, only 12 day-old biofilms were analyzed. ANOVA analyses showed a significance in the three-way interactions among substrate type, protective mesh and treatment type ($p=0.0101$; table 3.3). Similar to biofilm cariogenicity, the protective-mesh groups showed lower viability values when compared to non-mesh groups in both treatment types. The difference between substrate types was not significant (NaF $p=0.5$; DIW $p=0.7$).

3.4. Discussion

The main aim of this study was to evaluate the differences between human versus bovine enamel specimens as part of a microbial cariogenic model. A secondary aim was to evaluate the effectiveness of covering enamel samples with protective mesh to grow an even biofilm layer over the surface, and monitor its uniform growth up to 12 days. To our knowledge, this is the first study evaluating the differences in biofilm-induced carious lesions between enamel substrate types. Also, limited research studies have discussed interventions used to maintain growing a uniform biofilm layer over dental substrates.

Although there are well-established, widely used chemostat caries models,^{20, 70} the microbial component should be included in in-vitro caries models as a contributing factor in the initiation and progression of the disease. Creating a microbial caries model still has its challenges. A reliable, clinically relevant microbial model should allow the active attachment of biofilm to the surface.

The characteristics of the model should represent daily activities such as periodic changes in pH levels during the day; periodic exposure to sucrose, and periodic exposure to caries-preventive agents. Solutions used in the model should maintain bacterial viability and growth and also mimic daily challenges. In this study, we wanted to evaluate the variables mentioned earlier (protective mesh and substrate type) as essential components of a microbial caries model. This was a follow-up study to previous pilot experiments evaluating other factors such as: surface conditioning through the creation of acquired pellicle pre-biofilm growth; mineral saturation of growth media; and sucrose concentration (data not shown).

We chose the source of the microcosm bacterial biofilm to be pooled saliva from three donors. Collecting three samples, and having sucrose in our demineralization media, allowed overcoming any expected variability between the samples, as reported in the literature previously.^{41, 50-52} We allowed the biofilm to attach actively to the samples through mounting our samples on the inside of the lid of 24-well plates.²³

Another characteristic in the model we tested is the mesh. In a previous pilot study we conducted, it was visually apparent that the biofilm grown for an extended period of time does not grow evenly over the surface. It was reported previously by Mei et al. and other studies that the uneven distribution of the bacteria over the surface may result in larger variability in the results.^{48, 64-66}

We used a pH cycling model in this study. The model we used was modified from a previously published model.³² The use of a pH cycling model has its significance. It mimics daily activities, resulting in high clinical relevance. It is also applicable in-vitro, allowing the reproducibility of the model in future studies. We followed Zhang et al. (2015) protocol in the treatment types used in their study: 18.4 mM NaF (treatment) and DIW (negative control).³²

When looking at carious lesion severity, the actual values of the outcome analyses were different between human and bovine specimens. However, the pattern of lesion severity was still similar in most of the data (Table 3.1). A previous study by Lippert and Lynch (2014) evaluated the differences in carious lesions created on human and bovine samples.⁴⁶ The study used a chemostat caries model, whereas we incorporated the microbial component in our model. Our study was still consistent with the findings by

Lippert and Lynch (2014) which concluded that lesions in bovine enamel progress at a faster rate than human enamel.

Analyzing carious lesion severity (VHN_{change} , ΔZ , L) resulted in a significant interaction between pH cycling time, substrate type, and treatment type. However, the mesh factor was not a significant factor in the three or four-way ANOVA analyses. As the pH cycling time increases, an increased treatment effect was observed in both human and bovine samples.

We wanted to test the feasibility of using bovine enamel as a substitute to human enamel in cariology research (microbial models); bovine enamel is readily available and cost-effective.⁴⁶ As we found in this study, having similar patterns in biofilm cariogenicity and lesion severity with biofilm's maturation in both substrate types makes bovine enamel a suitable substitution to human enamel.

The outcome analyses we chose when testing biofilm's cariogenicity included biofilm virulence factors (LDH activities and EPS amounts). In general, testing biofilm virulence is more significant than biofilm's viability as an indicator of the effectiveness of anticaries/antibacterial compounds; this is because the main goal of using these compounds is interference with biofilm's cariogenicity, especially that killing the bacteria within the biofilm requires high concentrations of fluoride compounds.^{9, 18, 19, 60, 61, 71, 72}

When comparing substrate types, differences in normalized LDH and EPS values were observed between human and bovine specimens at some timepoints. However, the pattern of biofilm cariogenicity over time was similar among the two substrate types (Table 3.2, 3.3), which is consistent with lesion severity data.

In both human and bovine groups (non-mesh groups), a trend of an increased biofilm cariogenicity (i.e. normalized LDH and EPS values) was observed. In earlier biofilm (4D), there was no statistically significant difference between substrate types (Table 3.2). More mature biofilm behaved differently according to substrate type: the statistically significant difference started in 8-day biofilm EPS data. One can argue that since there was a significant difference between substrate types we should always use human specimens to maintain clinical relevance. Our study is the first study to explore substrate type as part of a microbial cariogenic model. Since the pattern of biofilm cariogenicity over time found in this study was the same between the two substrate types, using bovine specimens in microbial studies (especially studies exploring biofilm maturation) is still valid. Moreover, within the context of this study, if a future study does not evaluate biofilm maturation, the selection of substrate type can be based then on the study's duration.

When evaluating the application of a protective mesh, the results of biofilm analyses exhibited a significant effect of the protective mesh. All groups that included a protective mesh demonstrated a lower biofilm cariogenicity (i.e. LDH activity, EPS amounts, and viability).

The main purpose of covering the specimens with mesh was to create niches for the biofilm to attach to, and allowing the biofilm to be adherent to the surface without dispersal/detachment, especially considering that biofilm maturation was a variable evaluated in this study (up to 12 days). In the "mesh-groups", the values of LDH activity and EPS amounts were not only low when compared to non-mesh groups, but also continued to be low among all timepoints (up to 12 days). When we collected the biofilm

at each time-point, we used clean tweezers to remove the mesh first, as we were interested in analyzing the biofilm formed directly over the enamel surfaces. Then we loosened the specimen (using tweezers) and placed it in sterile saline for further analyses. Although we made sure not to disturb the biofilm layer formed over the sample itself, we believe that the physical removal of the mesh resulted in removing a large portion of the biofilm grown over enamel surface. This may explain the low cariogenicity values when compared to non-mesh groups.

Based on the results of our study, we believe that applying a protective mesh was not a practical solution, even though it ensures the growth of a more evenly distributed biofilm layer (evaluated visually). Zaro ⁷³ (1995) emphasized the importance of biofilm's thickness and the influence of this factor on lesion severity as well as the pattern of lesion's remineralization (when the biofilm serves as a diffuser to different ions). Zero (1995) compared gauze-free and gauze-covered enamel samples in in-situ experimental models. He concluded that applying the gauze, even if it resulted in less clinical relevance, is critical; it allows the creation of a thicker biofilm and higher demineralization/ remineralization to the enamel surface, and therefore should be explored more.⁷³. This is a great area of research and exploration in the future (i.e. finding interventions to maintain an even, thicker, actively attached biofilm in studies that extend to longer periods).

This study allowed a better understanding of the components of a controlled, more clinically-relevant, in-vitro microbial model. It established a further step to achieve an optimum microbial model. The large variability in the results has been a challenge in previous studies and was still a limitation to our study, and needs to be addressed. Future

studies may include the modification of the substrate's surface, such as omitting the polishing step or creating niches on the surface. Other studies may include a more detailed analysis of the substrate surface to overcome the large variability. This can be achieved by obtaining multiple VHN_{change} readings from different areas on the enamel surface, and analyzing two or more enamel sections for ΔZ and L .

3.5. Conclusion

In conclusion, within the study's limitations, human or bovine enamel specimens can be used in a microbial in vitro caries model to study biofilm's maturation and the effect of anticaries agents. The use of utility preceptive mesh over the samples prevents accurate evaluation of the biofilm that is formed over the enamel surface, and therefore should be avoided.

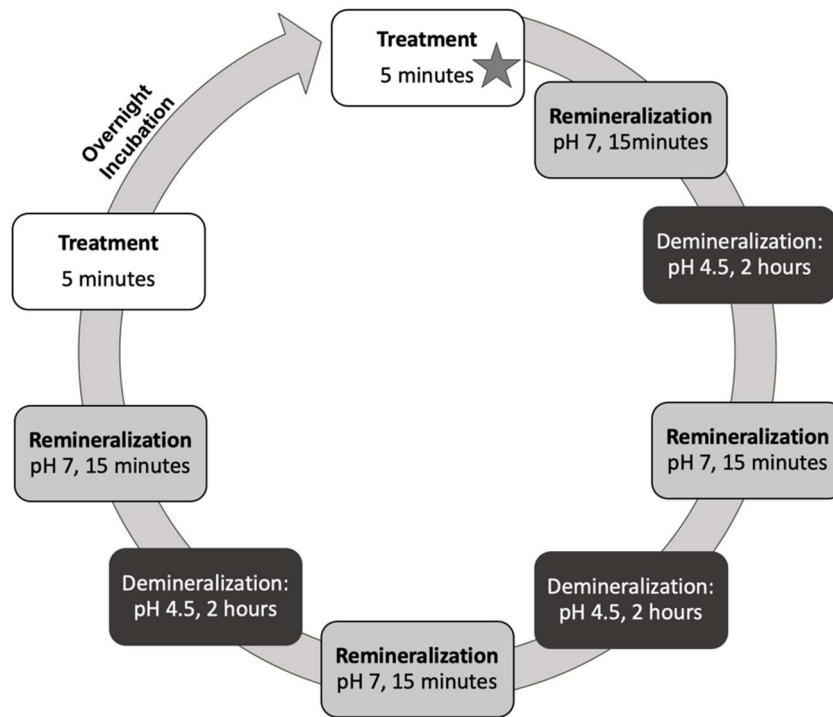


Figure 3.1. Daily pH cycling podel

Table 3.1. Caries lesion severity: surface microhardness change (VHN_{change}); integrated mineral loss (ΔZ ; %volmin $\times\mu\text{m}$); and lesion depth (L; μm). All data are presented as [mean (standard deviation)] as a function of maturation stage and treatment

		VHN _{change}			ΔZ			L		
		4 Days	8 Days	12 Days	4 Days	8 Days	12 Days	4 Days	8 Days	12 Days
Human	NaF	6.9	31	40.8	658	1156	828	44.3	85.6	46.1
		(5.8)	(16.6)	(16.1)	(601)	(551)	(619)	(50)	(52)	(48.2)
	DIW	15.8	67.2	74.2	1216	1092	1686	78.2	49.6	67.2
		(6.8)	(16.6)	(8.9)	(676)	(584)	(771)	(40.3)	(29.5)	(25.7)
Bovine	NaF	15.7	39.6	31.6	792	509	963	40.9	24.3	51.3
		(6.1)	(16.5)	(12.9)	(608)	(454)	(472)	(50)	(26.4)	(36.9)
	DIW	35.6	57	76	928	1513	2162	42.5	82.2	100.3
		(13.3)	(18.5)	(7.7)	(721)	(578)	(883)	(46.4)	(47)	(44.1)

Lower Case: significance among pH cycling duration (4 Days; 8 Days; 12 Days). Different lower case indicate significance between groups.

Upper Case: significance among treatment type (NaF; DIW). Different upper case indicate significance between groups

Numbers: significance among substrate type (Human; Bovine). Different numbers indicate significance between groups

Table 3.2. Biofilms cariogenicity: LDH activity (µg/ml), EPS amount (µg/ml), and viability. All data are presented as [mean (standard deviation)] as a function of maturation stage and treatment. All LDH activity and EPS amount values were normalized using protein concentration data

			LDH Activity Mean (SD)						EPS Amount Mean (SD)								
			4 Days			8 Days			12 Days			4 Days			8 Days		
Human	Mesh	NaF	1.5 (0.7)		1.9 (0.7)		3.3 (1.9)	A,◇	2 (0.6)		4 (1.3)		3.5 (2.1)		◇		
		DIW	1.7 (0.5)	a	1.5 (1.1)	a,◇	9.1 (3.7)	b,B,1,◇	2.5 (0.3)	a	2.2 (1)	a,◇	5.9 (1.9)	b,1,◇			
	No Mesh	NaF	1.4 (1.2)	a	2.9 (1.2)	a,A	14.5 (7.2)	b,A,1,◇◇	2.7 (0.7)	a	4.7 (2.5)	a,A,1	10.7 (3.7)	b,A,1,◇◇			
		DIW	0.6 (0.1)	a	7.6 (2.3)	b,B,◇◇	18.8 (6)	c,B,1,◇◇	2.7 (1.2)	a	7.9 (3.8)	b,B,◇◇	13.3 (6.8)	c,B,◇◇			
Bovine	Mesh	NaF	1 (0.3)		0.9 (0.7)	◇	1.3 (0.3)	◇	3.7 (2.2)		2.1 (0.6)	◇	2.9 (0.7)				
		DIW	0.9 (0.6)		1.7 (0.6)	◇	0.9 (0.2)	◇,2	2.3 (0.7)		4 (1.2)	◇	1.7 (0.4)	2,◇			
	No Mesh	NaF	0.9 (0.3)	a	4.8 (2.6)	b,◇◇	6.5 (2.1)	b,A,2,◇◇	2.7 (0.4)	a	10.2 (7.1)	b,A,2,◇◇	5.4 (1.2)	c,A,2			
		DIW	0.6 (0.2)	a	6.3 (2.3)	b,◇◇	10.1 (1.8)	c,B,2,◇◇	1.6 (0.5)	a	6.8 (3.1)	b,B,◇◇	11.1 (3.9)	c,B,◇◇			

Lower Case: significance among pH cycling duration (4 Days; 8 Days; 12 Days). Different lower case indicate significance between groups.

Upper Case: significance among treatment type (NaF; DIW). Different upper case indicate significance between groups

Numbers: significance among substrate type (Human; Bovine). Different numbers indicate significance between groups

Symbols: significance among the use of protective mesh (yes/no). The presence of ◇ and ◇◇ indicate significance between the two groups

Table 3.3. Biofilm viability (12 days-old biofilms)

		log₁₀ CFU/ml Mean (SD)		
Human	Mesh	NaF	9.4 (0.7)	A, ◇
		DIW	9.7 (0.2)	B,1, ◇
	No Mesh	NaF	10.1 (0.2)	◇◇
		DIW	10 (0.1)	◇◇
Bovine	Mesh	NaF	9.2 (0.2)	A, ◇
		DIW	8.8 (0.3)	B,2, ◇
	No Mesh	NaF	10 (0.1)	◇◇
		DIW	9.9 (0.1)	◇◇

Upper Case: significance among treatment type (NaF; DIW).
Different upper case indicate significance between groups
Numbers: significance among substrate type (Human; Bovine).
Different numbers indicate significance between groups
Single and Double Diamond Symbols: significance among the
use of protective mesh (yes/no). The presence of ◇ and ◇◇
indicate significance between the two groups

CHAPTER 4: THE ANTI-CARIES EFFICACY OF THREE FLUORIDE COMPOUNDS AT INCREASING MATURATION OF A MICROCOSM BIOFILM

4.1. Introduction

Dental plaque (biofilm) plays a critical role as one of several factors in the initiation and progression of carious lesions.² When dental biofilm adheres to tooth structures for a prolonged period of time, it metabolizes dietary carbohydrates through glycolysis - this leads to lactic acid production and therefore a drop in pH levels in the oral cavity.⁷ The ability to produce and tolerate acids (acidogenicity and acidity, respectively) is considered a key virulence factor for the survival of the biofilm.¹⁹ Another key feature is the biofilm's ability to utilize dietary carbohydrates to produce glucosyltransferases (*Gtfs*) and synthesize water-insoluble extracellular polysaccharides (EPS). All these virulence factors cause a shift in the balance of oral bacteria to become cariogenic.¹⁹

Mechanical removal of dental biofilm is an effective way to break the caries process. However, biofilm starts to reform immediately afterwards. Many studies have been conducted to test the efficacy of antimicrobials against biofilms cariogenicity in terms of virulence factors.^{6, 19, 74, 75} Fluoride has been documented as the gold standard anticaries agent.¹¹ Fluoride is known for its effect in enhancing remineralization and preventing demineralization of tooth structures. An additional anticariogenic effect of fluoride is its effect on the virulence of the biofilm: acidogenicity, acidity, and *Gtfs* secretion, making biofilm's virulence a target to break the caries process.⁷⁴

In toothpastes, fluoride can be found in many forms such as sodium fluoride (NaF), sodium monofluorophosphate, stannous fluoride (SnF₂), and amine fluoride

(AmF).¹² Previous studies have shown that SnF₂-containing toothpastes were able to significantly reduce dental plaque and affect biofilm's architecture.^{76, 77} This antibacterial effect of SnF₂ can be attributed to the stannous ion through its ability to reduce bacterial acidogenicity and glucan production.⁷⁸ Exterkate, et. al. (2010) evaluated the antibacterial effect of AmF and found it to be a dose-dependent effect. This effect was significantly influenced by the nature of the biofilm (i.e. single-species vs. microcosm biofilm), indicating that all these factors should be considered when studying the efficacy of AmF-containing oral care products.²³

As the biofilm continues to grow on oral/dental surfaces (i.e. matures), changes occur within the biofilm in terms of dominance of acidogenic bacteria.¹ Therefore, we expect that at different maturation stages, an alteration in the interaction between biofilm and antimicrobial agents can be observed. Since there are many phenotypic changes observed within biofilm, and since these changes are suggested to be factors giving the biofilm its ability to tolerate microbial agents,¹⁶ testing the interaction between biofilm and these agents at each stage is necessary to obtain an optimum cariostatic effect against oral bacteria.¹⁶

Previous studies have explored the biofilm at different ages. However, these studies focused on single, two or multiple-species biofilms.^{1, 23, 24} Most of these studies limited their experiments to testing biofilm at one or two maturation stages.^{1, 2, 23, 24} As there is considerable scope for further research, the aim of our study was to explore the anti-carries efficacy of three fluoride compounds, NaF, SnF₂, AmF, at increasing maturation stages of a microcosm biofilm grown on bovine enamel. We tested, at each maturation stage, the carious lesion severity as well as the cariogenicity of the biofilm.

4.2. Materials and Methods

Study Design

Microcosm biofilm obtained from human saliva was grown with active attachment on bovine enamel specimens for 24 hours using Brain Heart Infusion (BHI) media supplemented with 0.2% sucrose. The model was pH cycled, where it was exposed to two 5-minute treatment periods (treatments used were NaF, SnF₂, AmF, and de-ionized water [DIW]), three 2:15-hour demineralization challenges, and four 10-minute remineralization periods. The pH cycling continued for either 4 days (4D), 8 days (8D), or 12 days (12D). At the end of each pH cycling period, enamel specimens were analyzed for caries lesion severity: surface microhardness (VHN_{change}); transverse microradiography (integrated mineral loss [ΔZ] and lesion depth [L]). The biofilm was collected and analyzed for its cariogenicity: lactic acid production (LDH activity), exopolysaccharide (EPS) amount, and viability (log₁₀ CFU/ml). The experiment was repeated three times.

Specimen Preparation

For this study, 216 enamel specimens (5 x 5 mm) were obtained from extracted bovine incisors, which were sectioned using Buehler IsometTM low-speed saw (Buehler, Ltd., Lake Bluff, IL, USA). To ensure ensure flat parallel dentin/enamel surfaces, all specimens were ground and polished using a Struers Rotapol 31/Rotoforce 4 polishing unit (Struers Inc., Cleveland, PA, USA). Then, the dentin side was ground using 500-grit silicon carbide grinding paper. For the finishing step, the enamel side was subject to grinding using 1,200, 2,400 and 4,000 grit papers. After that, polishing of the specimens

took place using a 1 μm diamond polishing suspension on a polishing cloth to obtain a 5 \times 5 mm polished enamel surface. Specimens were checked for cracks, white spots, or flaws that lead to excluding the specimen from the study, using a Nikon SMZ 1500 stereomicroscope at \times 20 magnification. During preparation, the teeth were stored in deionized water with thymol.

Baseline Measurement and Experimental Groups

Surface microhardness testing ($\text{VHN}_{\text{sound}}$) was used as the baseline measurement. A Vickers diamond identifier (Tukon 2100; Wilson-Instron, Norwood, MA, USA) was used with a load of 200 g for 15 s. Three indentations, approximately 100 μm apart, were created on each specimen and averaged; the inclusion range was $\text{VHN}_{\text{sound}}$ between 300-380.

Specimens were divided then into three groups, based on the pH cycling duration (4D, 8D, and 12D). Each group had four subgroups ($n=18/\text{subgroup}$), based on the treatment type: NaF, SnF_2 , AmF, and DIW). All three fluoride compounds were used at a concentration of 287.5 ppm in deionized water (prepared fresh daily, used without pH adjustment), simulating a fluoride concentration of 1150 ppm found in toothpastes after a 1:3 dilution occurring during toothbrushing.

An active attachment model, following a previously described protocol.²³, was used in this study: specimens were mounted on the inside of a lid of a 6-well plate (FisherBrand, Fisher Scientific) using acrylic cubes. The model was disinfected using 70% ethanol prior to biofilm growth.⁴⁰

Salivary Bacterial Model

Saliva Collection

The approval for saliva collection was obtained from the IUPUI institutional review board (IRB #1406440799). The inclusion criteria for saliva donors included: with normal salivary flow and no systemic diseases or active caries or periodontal diseases. Participants refrained from oral hygiene measures overnight. Three donors participated by providing wax-stimulated saliva samples (approx. 50 ml/donor). Samples were then pooled and tested for the presence of *Streptococcus mutans* and *Lactobacilli* using selective agars. The results confirmed the presence of both species.

Five ml of the pooled saliva and growth media mix (1:10 ratio) were incubated overnight, then mixed with 10% glycerol and frozen immediately at -80° C, this microcosm bacterial mix was used as the source for bacterial inoculum.

Biofilm Growth

The biofilm was allowed to grow on the enamel specimens for 24 hours at 5% CO₂ and 37° C in the growth media. The growth media for this model contained Brain Heart Infusion (BHI) broth, supplemented with 5 g/l yeast extract (YE), 1 mM CaCl₂·2H₂O and 0.2% sucrose. After 24 hours, pH cycling protocol started, as illustrated in Figure 4.1.

pH cycling Model

After the 1-day biofilm growth on enamel specimens, the biofilm model was subjected to a cariogenic pH cycling model, where the growth media was used as both the

remineralization (remin) and demineralization (demin) solutions (Figure 4.1). Both the remin and demin media contained BHI broth, 5% YE, 1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.

Using 1 mM acetic acid, the pH of culture medium was adjusted to 7 (remin), and 4.5 (demin). The sucrose concentration also differed in the remin (no sucrose) and demin (1% sucrose) solutions. Between treatments, the biofilm was washed by immersing the model in 0.9% sterile saline for 2 minutes. The pH cycling model was conducted daily, the biofilm and specimens were incubated overnight in remin media.

At the end of each pH cycling period (4D, 8D, 12D), biofilm collection took place by carefully removing biofilm-covered enamel specimens using tweezers, then placing each specimen in an Eppendorf tube containing 1 ml sterile saline; sonicating at 30 W for 10 seconds, then vortexing immediately for 10 seconds for complete biofilm detachment from the enamel surface. The experiment was repeated three times; the same source for bacterial inoculum was used in all three experiments.

Post-treatment Analysis

Enamel Substrate

- Surface Microhardness Change ($\text{VHN}_{\text{change}}$)

Surface microhardness was measured post-treatment following the same protocol used for the $\text{VHN}_{\text{sound}}$ values. The $\text{VHN}_{\text{change}}$ values were calculated using the formula $\text{VHN}_{\text{change}} = 100 * (\text{VHN}_{\text{sound}} - \text{VHN}_{\text{post}}) / \text{VHN}_{\text{sound}}$.

- Transverse Microradiography

Silverstone-Taylor Hard Tissue Microtome (Scientific Fabrications Laboratories, USA) was used to obtain an approx. 100 μm thick section from each specimen, which was cut from the center of each specimen and across the specimen. Sections were placed in the TMR-D1 v.5.0.0.1 system and X-rayed at 45 kV and 45 mA at a fixed distance for 12 s. An aluminum step wedge was X-rayed under identical conditions. All captured images were analyzed using TMR software v.3.0.0.18. Sound enamel was assumed to be 87% v/v mineral. The data obtained from this analysis were integrated mineral loss (ΔZ) and lesion depth (L).

Biofilm Analysis

- Lactic Acid Production: LDH Assay

In order to determine live biofilm metabolism, lactic acid production was assessed using a LDH cytotoxicity assay, following a previously published protocol.⁶⁷ For each sample, 45 μl of the collected, suspended biofilm was mixed with 5 μl of the LDH Assay Lysis Solution in a 96-well microtiter plate, and incubated at 37° C for 45 minutes. 100 μl of LDH Assay mixture was added to the cell lysate (LDH Assay Cofactor Preparation: LDH Assay Substrate: LDH Dye Solution = 1:1:1). The mixture was kept in the dark and incubated at room temperature for 30 minutes. To terminate the reaction, 50 μl of Stop Solution was added to the mixture. We measured the LDH absorbance readings at OD_{490nm} and the background absorbance at OD_{690nm}. The background absorbance values were subtracted from the LDH readings.

- EPS amount: Phenol-Sulfuric Acid Colometric Assay

Analyzing the EPS activity was performed using a previously described protocol.

⁶⁸ Briefly, 50 µl of the biofilm suspension of each sample was transferred to a 96-well microtiter plate. For each sample, 150 µl of concentrated sulfuric acid was added. Immediately after that, 30 µl of a 5% phenol solution was added to the mixture and heated to 90° C for 5 minutes. After cooling the plate in room temperature for 5 minutes the absorbance was measured at OD_{750nm}.⁶⁸

All LDH and EPS values were normalized by the protein concentration of each biofilm sample, using a RC DC Protein Assay (Bio-Rad Laboratories, Inc. Hercules, CA, USA) following the manufacturer's instructions.

- Bacterial Viability

At the end of each time point, biofilm samples from all groups were serially diluted to 1:10⁻³, 1:10⁻⁴, and 1:10⁻⁶ dilutions (using 0.9% sterile saline). Samples were plated on Blood Agar Plates (Thermo Scientific™, Remel, Lenexa, KS, USA) using a Spread Plate Procedure.⁶⁹ To determine bacterial counts, mean log₁₀ CFU/ml values were calculated.

Statistical Analysis

All post-treatment analyses were analyzed using two-way ANOVA, with factors for pH cycling duration (4D, 8D and 12D) and treatment type (NaF, SnF₂, AmF, and DIW) as well as the interactions between them to test the effect of pH cycling duration and treatment type. All pair-wise comparisons from ANOVA analysis were made using

Fisher's Protected Least Significant Differences to control the overall significance level at 5%.

4.3. Results

Specimen image 1 shows biofilm-coated enamel surfaces on the day of collection at each time point, treated with 4 different treatments.

Dental Substrate - carious lesion severity

The ANOVA analysis tested the interaction between the two variables explored in this study. However, the interaction was only significant for the VHN_{change} ($p=0.0354$) but not for the TMR data (L: $p=0.2412$; ΔZ : $p=0.6811$). The anticaries effect of SnF_2 and AmF was higher than that of NaF and DIW for all variables (i.e. VHN_{change} , L, and ΔZ). (Table 4.1)

The DIW groups exhibited a slight, non-significant increase in carious lesion severity with increased biofilm maturation. However, this trend was not consistent with what was found in any of the fluoride-treated groups.

Regarding VHN_{change} data, no significant differences were observed as the biofilm matured (except between 4D and 8D in groups treated with NaF and SnF_2). Within each maturation stage, all fluoride compounds showed a statistically significant higher VHN_{change} values than DIW.

The transverse microradiography analysis (L and ΔZ) demonstrated that the time factor did not lead to a significant difference in any of the treatment types. Within each maturation stage, all fluoride compounds showed statistically significant lower L and ΔZ

values than DIW. However, there was no statistically significant difference between the three fluoride compounds.

Biofilm

Table 4.2 shows the results of the biofilm analyses. The ANOVA analysis tested the interaction between the two variables explored in this study - biofilm maturation and treatment type. It showed a significant interaction for LDH activity, EPS activity, and viability ($p<.0001$; $p<.0001$; $p=0.0001$, respectively).

In general, almost all groups showed a trend of an increased biofilm cariogenicity as the biofilm matures. Also, all fluoride-treated biofilm showed less cariogenicity (i.e. LDH and EPS activity) when compared to DIW groups.

Both LDH and EPS activities showed similar trends. The less mature biofilm (i.e. 4 days old) did not show a significant difference between any treatment types (all p values were >0.05). However, with more mature biofilm, the anticaries effect of SnF_2 and AmF continued to be significantly higher than for NaF and DIW.

Although the anticaries effect of NaF was not significantly different than the DIW group in earlier biofilm (4D and 8D), a significant difference between those two groups occurred at 12D, indicating that the repeated exposure to NaF over 12 days may cause a significant anticaries effect, when compared to DIW.

SnF_2 and AmF also showed the highest effect in bacterial viability data (presented as \log_{10} CFU/ml) in all groups (AmF showed a significantly higher effect than SnF_2 in 8D and 12D groups). NaF did not show a statistically significant difference compared to DIW groups in any of the maturation stages. The 12D NaF and DIW groups showed a statistically significant higher viability values than the 4D groups.

4.4. Discussion

The main objective of our study was to determine changes in anticaries efficacy of the three fluoride compounds tested at increasing maturation stages of a microcosm biofilm. We determined these changes by analyzing both the cariogenicity of the biofilm as well as the extent of demineralization in the enamel specimens. To our knowledge, this is the first study focusing on how changes in the characteristics of the biofilm due to maturation may alter the anticaries potential of fluoride.

Fluoride can affect biofilm cariogenicity due to its ability to interfere with the biofilm virulence factors such as acid production and EPS synthesis⁷⁵ Acid production, which causes dissolution of dental structures, is considered the most virulent factor in the biofilm.^{79, 80} EPS, which is also produced by the bacteria within the biofilm, serves as a defense mechanism, protecting the biofilm against antimicrobials. It also serves as a reservoir for nutrients to maintain bacterial viability.^{7, 9, 61, 63, 81}

To measure the severity of the carious lesions formed, we used both surface microhardness and transverse microradiography to determine changes in enamel structure and mineral content. All fluoride compounds were able to produce a preventive effect, in terms of carious lesion severity, when compared to DIW. However, no differences were observed between fluoride compounds. Moreover, DIW groups showed a non-significant trend of increased carious lesion severity (ΔZ and L data) with increased biofilm maturation. A possible explanation for the absence of this trend in fluoride treated groups could be related to our findings regarding biofilm data. Since the biofilm continued to be viable even with daily fluoride exposure, the biofilm may have served as a reservoir of fluoride. A very important role of intra-oral biofilms is their ability to retain minerals,

such as fluoride and calcium.^{32, 82, 83} In future studies, analyzing the fluoride content within the biofilm as a function of depth⁸⁴ may shed some light on the relative ability of the tested fluoride compounds to deliver fluoride to biofilms of varying maturity.

The results of the LDH and EPS activities demonstrated altered tolerance levels (i.e. LDH and EPS activities) of biofilms based on treatment types and maturation stage. At each maturation stage, NaF showed the least anticaries efficacy when compared to SnF₂ and AmF. When comparing maturation stages, biofilms were more tolerant to NaF and controls, while SnF₂ and AmF continued to produce a higher anticaries effect over time. These findings are consistent with previous studies that evaluated the antimicrobial efficacy of NaF and found, both in vitro and in vivo, that NaF lacks a sustainable antimicrobial effect.^{85, 86}

None of the fluoride compounds showed complete bactericidal effects at the concentrations tested. This finding was expected as it is consistent with previous studies.^{1, 23, 32, 87} The concentration of fluoride compounds used in this study was 287.5 ppm, which mimicked the dilution of 1150 ppm fluoride found in toothpastes during toothbrushing (1:3 dilution ratio). A previous study found that concentrations greater than 225 ppm fluoride as NaF are needed for complete inhibition of *S. mutans* biofilm formation.²⁹ Given the more complex and more mature biofilm used in our study, fluoride concentrations used in our study are unlikely to display a bactericidal effect. In general, the ultimate goal of using fluoride as a caries-preventive agent is inhibiting the cariogenicity of the microcosm biofilm rather than reaching a full bactericidal effect.²³

In vitro caries models can be, albeit crudely, divided into those involving a human dental plaque analogue and those that do not. The most common and well-established

models,^{20, 70} do not incorporate the microbial component as a contributing factor in carious lesion formation and utilize acidic solutions to mimic biofilm-derived cariogenic acids. In our study, we attempted to create an in-vitro microbial caries model which allows studying biofilms in conditions that achieve more clinical relevance. Previous studies tested several microbial caries models to mimic the complexity of the oral cavity. These models vary from simpler ones such as microtiter plates, to more complex models, such as constant depth film fermenters and artificial mouth models.^{2, 88-91} However, more research is still needed in order to optimize the conditions to create a well-established in-vitro microbial caries model. Some characteristics of an ideal model may include: creating the active attachment of bacteria to the surface, maintaining viability and growth, and also allowing high-throughput screening.²³ In our study, we used an active attachment model to ensure the attachment of the bacteria within the biofilm to the enamel surface, rather than an accumulation of sedimented layers of bacteria over the surface. Likewise, models are often overestimating in vivo caries development and progression as caries lesions are often stagnant and only very few progress to cavitation.⁹²

The pH cycling used in this study was modified from a previously published model.³² To test its applicability in creating a representative, microbial-induced carious lesion, we evaluated our model through conducting pilot studies (data not shown). We optimized the demineralization and remineralization pH levels, pH cycling periods, and growth media conditions (i.e. mineral saturation). Based on these preliminary data, we chose the conditions used in this study. We used in our study pooled saliva collected from three donors as our source of the microcosm biofilm. One could suggest that using a microcosm biofilm source may result in large variability. The variability of biofilm

characteristics in in-vitro studies was explored previously.⁴¹ Most studies^{50 51 52} concluded that collection of saliva samples from the same donor at different times did not affect biofilm diversity. Moreover, other studies⁴¹ stated that the involvement of sucrose over time can lead to a dominance of certain bacterial strains (mainly cariogenic bacteria), thus overcoming initial differences between different samples (either from different donors or collected at different times from the same donor).

Bovine specimens were used for biofilm growth. Although using human enamel specimens may seem more clinically relevant, carious lesions formed in bovine enamel have similar characteristics except that lesions created in bovine teeth progress faster than in human teeth.⁴⁶ Bovine enamel is cost-effective, readily available and given the increasing difficulty in obtaining human teeth suitable for caries research, bovine teeth may become the go-to substrate in the future.

The growth media we used was BHI growth media, supplemented with several minerals and nutrients to maintain bacterial viability. Similarly, a previous study used growth media as the demineralization/remineralization buffer. They used HEPES as a buffer in their media to maintain high pH (except during demineralization periods). Their study tested a pH cycling model with and without biofilm to evaluate the biofilm as a diffusion barrier to antimicrobials.³² Because our aim was to create a biofilm-induced carious lesion, we allowed the lactic acid produced by the bacteria to create an effect. However, we measured the pH level of the overnight media every morning. Although it dropped from pH 7, the pH level was maintained above 5.5 for all treatment groups (data not shown).

Previous studies that investigated the microbial component as part of the caries process either studied a single or two maturation stages, or focused on one, two, or several microbial species.^{1, 29, 48} One of the main objectives of this study was focused on testing several maturation stages and the characteristics of each maturation stage. We also ensured the use of human saliva as our source of bacterial biofilm to maintain clinical relevance.

This study did not evaluate phenotypic changes nor bacterial dominance as the biofilm matured. Based on the main aim of the study, we only focused on analyzing the biofilm's cariogenicity and not the fluoride-induced alterations in biofilm, which will be the subject of future studies. Another area that was not explored in this study was the effect of varying fluoride concentrations. We limited our study to fluoride concentration that corresponds to what is in over-the-counter toothpastes. Testing a range of fluoride concentrations of each compound would be valuable to evaluate the optimum needed for caries prevention.

Considering the present limitations and study conditions, the findings of our study provide a better understanding of biofilm maturation and the impact of this important factor on altering the cariostatic effect of fluoride compounds. Findings from this study serve as basic knowledge for future studies and possibly clinical applications in the future, such as in patients expected to have more mature biofilm/plaque in stagnation areas in the oral cavity (e.g. special needs patients or orthodontic patients with hard-to-reach areas). Future studies may include testing the recovery of biofilm cariogenicity after prolonged exposure to fluoride treatments.

4.5. Conclusion

Biofilm maturation plays a very important role in increasing biofilm's tolerance against fluoride treatment. Also, the maturation stage of biofilm could influence the selection of fluoride compounds to achieve an optimum cariostatic effect.

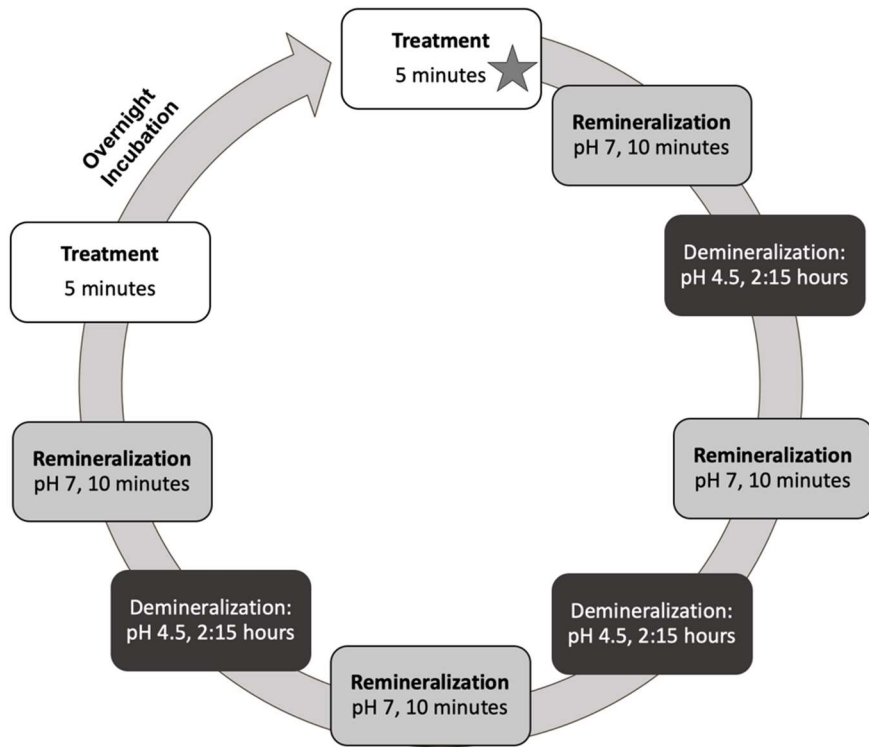


Figure 4.1. Daily pH cycling protocol

Table 4.1. Caries lesion severity: surface microhardness change (VHN_{change}); integrated mineral loss (ΔZ ; vol%/min $\times\mu\text{m}$); and lesion depth (L; μm). All data are presented as [mean (standard deviation)] as a function of maturation stage and treatment

	VHN_{change}						ΔZ						L					
	4 Days		8 Days		12 Days		4 Days		8 Days		12 Days		4 Days		8 Days		12 Days	
NaF	59 (14)	a,A	68 (18)	b,A	60 (28)	a,A	1014 (587)	A	1192 (570)	A	1121 (699)	A	46.2 (22.7)	A	53.7 (18.9)	A	55.6 (43.6)	A
SnF₂	48 (17)	a,B	57 (19)	b,B	55 (26)	b,AB	988 (769)	A	1023 (543)	A	847 (521)	A	52.6 (40.4)	A	57.6 (25.0)	A	54.7 (41.5)	A
AmF	56 (15)	A	56 (21)	B	52 (27)	B	1008 (523)	A	865 (519)	A	904 (502)	A	50.1 (22.3)	A	40.6 (23.4)	A	45.2 (28.2)	A
DIW	83 (14)	C	83 (16)	C	80 (14)	C	2431 (1279)	B	2659 (1360)	B	2717 (1271)	B	83.7 (36.4)	B	99.7 (36.3)	B	108.4 (28.7)	B

Lower case: significance among pH cycling durations

Upper case: significance among treatment types

	LDH Activity						EPS Amount						Viability					
	4 Days		8 Days		12 Days		4 Days		8 Days		12 Days		4 Days		8 Days		12 Days	
NaF	0.5 (0.2)	a	2.7 (1.3)	b,A	4.3 (3.6)	c,A	1.7 (0.6)	a	3.9 (3.3)	b,A	7.5 (5)	c,A	7.3 (0.3)	a,A	7.7 (0.2)	a,b,A	8.2 (0.7)	b,A
SnF2	0.3 (0.3)		0.4 (0.1)	B	0.3 (0.2)	B	1.5 (0.4)		1.7 (0.7)	B	2.6 (1.1)	B	5.9 (0.1)	a,B	5.1 (0.6)	b,B	7.0 (0.6)	c,B
AmF	0.2 (0.1)		0.2 (0.6)	B	0.3 (0.2)	B	2.2 (0.9)		2.2 (1.0)	B	2.3 (0.7)	B	5.4 (0.5)	a,B	4.1 (0.2)	b,C	5.7 (0.6)	a,C
DIW	0.8 (0.7)	a	2.3 (1.6)	b,A	5.4 (2.4)	c,A	2.1 (1.3)	a	4.0 (2.6)	b,A	11.7 (5.1)	c,A	7.1 (0.4)	a,A	7.6 (0.5)	a,A	8.5 (0.5)	b,A
Lower case: significance among pH cycling durations Upper case: significance among treatment types																		

CHAPTER 5: THE INFLUENCE OF BIOFILM MATURATION ON THE ANTI-CARIES EFFICACY OF THREE FLUORIDE COMPOUNDS

5.1. Introduction

Dental biofilm is an essential factor for the initiation and progression of carious lesions. The ability of bacterial biofilm to produce lactic acid as an end product of the glycolytic pathway is the key virulence factor related to caries.^{6, 7} Therefore, disrupting the ability of the biofilm to produce acid is one of the approaches to control dental caries. Another virulence factor related to the caries process is the formation of the matrix, of which extracellular polysaccharides (EPS) are the main component.^{7, 9, 10} EPS serve as a protective environment for the bacteria; they store nutrients when they become scarce in the environment, facilitate acid formation, and also serve as a diffusion barrier against antibacterial agents.^{9, 10, 18, 61, 63}

The interaction between biofilms and different fluoride compounds has been explored previously.^{1, 6, 7, 19, 23, 28, 32, 48, 71, 74, 75, 77, 78, 85, 86} However, the impact of biofilm maturity (i.e. age) and how it affects the tolerance of the biofilm to fluoride compounds has not been studied before. Previous studies have focused mostly on biofilms at a certain age^{1, 23, 29} In the present study, we focused on the influence of timing of exposure to fluoride (i.e. early vs. late exposure) and biofilm maturation on the anticaries effect (i.e. on bacterial cariogenicity and carious lesion severity) of fluoride compounds.

Since the approach taken in most studies focused mainly on biofilm virulence factors and not bacterial viability^{6, 7, 17-19}, studying the biofilm recovery after fluoride treatment should also be taken into consideration. Some previous studies suggested that brief fluoride treatment through oral hygiene measures may allow the biofilm to recover

after a certain time.^{6, 10, 17} Hence, we aimed in our study to evaluate the long-term recovery after exposing the cariogenic model to pH cycling, which included fluoride treatments.

Therefore, the two main aims we explored in our study were: first, to explore the influence of biofilm maturation and time of exposure to fluoride treatments on the anticaries efficacy of fluoride; second, to explore the recovery of biofilm cariogenicity after exposure to fluoride treatments using two exposure periods.

5.2. Materials and Methods

Study Design

After specimen preparation, baseline hardness measurements, and saliva collection, the study was initiated by growing biofilm for 24 hours in Brain Heart Infusion (BHI) media (with 2% sucrose). Then, the pH cycling phase began. The number of days of pH cycling for each subgroup and type of treatment each day (i.e. fluoride compound or DIW) were determined based on the aim of the study (Figure 4.1 and Tables 5.1a and 5.1b).

For the first part of aim 1, the pH cycling continued for 4, 6, or 10 days, whereas in the second part, the biofilm in all subgroups was allowed to grow for 10 days. In both parts, each subgroup was subjected to a specific time of exposure to fluoride (i.e. T1, T2, or T3) (i.e. “exposure period”) and treatment types (sodium fluoride [NaF], stannous fluoride [SnF₂], amine fluoride [AmF], and de-ionized water [DIW]). Depending on the exposure period (T1, T2, or T3; exposure vs. non-exposure period) and cycling day, samples and biofilm were subjected to either fluoride treatment or just DIW (Figure 4.1).

The second aim of the study tested the ability of the biofilm to recover its cariogenicity after being exposed to fluoride for 4 days. In the first part, we allowed early exposure to

treatments (T1; days 1-4), then we collected the biofilm at three time points: immediate collection; 3-days post-treatment; 6-days post-treatment. In the second part, later exposure to fluoride treatments was conducted (T2: days 3-6). Then, two collection timepoints were selected: immediate collection and 4-days post-treatment.

During any period apart from exposure periods, pH cycling continued with substituting fluoride treatments with DIW. At any collection time, enamel specimens were analyzed for caries lesion severity using Vickers microhardness and transverse microradiography; the biofilm was collected and analyzed for its cariogenicity (lactate dehydrogenase enzyme [LDH] activity, EPS amount, and bacterial viability).

Specimen Preparation

Extracted bovine incisors were sectioned to obtain 5×5 mm enamel specimens using a Buehler IsometTM low-speed saw (Buehler, Ltd., Lake Bluff, IL, USA). During preparation, the teeth were stored in deionized water with thymol. Using a Struers Rotapol 31/Rotoforce 4 polishing unit (Struers Inc., Cleveland, PA, USA), all specimens were ground and polished to ensure flat parallel dentin/enamel surfaces. For the finishing process, the dentin side was ground using 500-grit silicon carbide grinding paper. Then, the enamel side was serially ground using 1,200, 2,400 and 4,000-grit papers. After that, polishing of the specimens took place using a 1- μ m diamond polishing suspension on a polishing cloth to obtain a 5×5 mm polished enamel surface. All specimens were checked for cracks, white spots, or any other flaws that could lead to excluding the specimen from the study, using a Nikon SMZ 1500 stereomicroscope at $\times 20$ magnification.

Baseline Measurement and Experimental Groups

A Vickers diamond identifier (Tukon 2100; Wilson-Instron, Norwood, MA, USA) was used with a load of 200 g for 15 s. The average VHN_{sound} measurement for each sample was obtained by creating three indentations, approximately 100 μm apart; the inclusion range was VHN_{sound} between 300-380.

We used an active attachment mode, following a previously described protocol.²³, l to allow true attachment between the bacterial biofilm and enamel specimens; specimens were mounted on the inside of a lid of a 6-well plate (FisherBrand, Fisher Scientific) using acrylic cubes. The model was disinfected using 70% ethanol prior to biofilm growth.⁴⁰

Specimens were divided then into three major groups, based on the timing of exposure period to treatments. In the early exposure period (T1), we exposed the samples to treatments on days 1-4, of pH cycling. The later exposure period (T2) allowed the exposure for days 3-6. The late exposure period (T3) allowed the exposure to treatments on days 6-10 of pH cycling. During the remaining days of pH cycling, fluoride treatments were replaced with deionized water. (Figure 5.1)

The first aim of the study included five collection timepoints (Table 5.1a). The first part (immediate biofilm collection), allowed collecting the biofilm coated samples on the following days: (T1: day 4; T2: day6; T3: day 10). The second part allowed full growth of the biofilm up to 10 days for all exposure periods.

The second aim tested the biofilm's recovery and compared it to immediate biofilm collection post-treatment (Table 5.1b); the collection timepoints were the following: (T1: days 4, 7, and 10; T2: days 6 and 10).

For each collection timepoint, four subgroups were included based on the treatment type: NaF (ACROS Organics, Fair Lawn, NJ), SnF₂ (ACROS Organics, Fair Lawn, NJ), AmF (GABA, Grabetsmattweg, Therwil, Switzerland), and negative control (deionized water; DIW). All three fluoride compounds were used at a concentration of 287.5 ppm in deionized water (prepared fresh daily, used without pH adjustment), simulating a fluoride concentration of 1150 ppm found in toothpastes after 1:3 dilution occurring during toothbrushing.

Salivary Bacterial Model

Saliva Collection

Microcosm biofilm was used in this study where the source is pooled wax-stimulated human saliva collected from three donors (ethical approval: IUPUI institutional review board [IRB #1406440799]). The inclusion criteria for the donors were: healthy participants (no systemic diseases) with normal salivary flow and no presence of active caries or periodontal disease. Participants refrained from oral hygiene measures overnight. Five ml of the pooled saliva and growth media mix (1:10 ratio) were incubated overnight, then mixed with 10% glycerol and frozen immediately at -80° C, this microcosm bacterial mix was used as the source for bacterial inoculum.

Biofilm Growth

Prior to pH cycling, we grew the biofilm on the enamel specimens for 24 hours at 37° C in a growth medium that contained Brain Heart Infusion (BHI) broth, 5 g/l yeast extract (YE), 1 mM CaCl₂.2H₂O and 0.2% sucrose.

pH cycling Model

In this model, the growth medium was used as both the remineralization (remin) and demineralization (demin) solutions (Figure 4.1). Both the remin and demin medium contained BHI broth, 5% YE, 1 mM CaCl₂.2H₂O.

The pH of culture medium was adjusted to 7 (remin), and 4.5 (demin) with 1 mM acetic acid. The remineralization media did not contain sucrose, while the demineralization media contained 1% sucrose. Sterile saline (0.9%) was used to wash the biofilm and enamel specimens between treatments; we immersed the model in the sterile saline for 2 minutes. We repeated the pH cycling model daily. Overnight, we incubated the model in remin media.

At the end of each pH cycling period, we collected the biofilm by carefully removing the biofilm-covered enamel specimens using tweezers. We then placed each specimen in an Eppendorf tube containing 1 ml sterile saline; sonicating at 30 W for 10 seconds, and vortexing immediately for 10 seconds for complete biofilm detachment from the enamel surface. The experiment was repeated three times; the same source for bacterial inoculum was used in all experiments.

Post-treatment Analysis

Enamel Substrate

- Percent Surface Microhardness Change (VHN_{change})

Post-treatment surface microhardness was measured following the same protocol used for the VHN_{sound} . The VHN_{change} values were calculated using the formula

$$VHN_{\text{change}} = 100 * (VHN_{\text{sound}} - VHN_{\text{post}}) / VHN_{\text{sound}}.$$

- Transverse Microradiography (TMR)

One section, approx. 100 μm thick, was cut from the center of each specimen and across the specimen using a Silverstone-Taylor Hard Tissue Microtome (Scientific Fabrications Laboratories, USA). All sections were placed in TMR-D1 v.5.0.0.1 system and X-rayed at 45 kV and 45 mA at a fixed distance for 12 s. An aluminum step wedge was X-rayed under identical conditions. Digital images were analyzed using TMR software v.3.0.0.18. Sound enamel was assumed to be 87% v/v mineral. The data obtained from this analysis were integrated mineral loss (ΔZ) and lesion depth (L).

Biofilm Analysis

- Lactic Acid Production: LDH Assay

In order to determine the live biofilm metabolism, the lactate dehydrogenase enzyme activity was determined using a LDH cytotoxicity assay, following a previously published protocol.⁶⁷ For each sample, 45 μl of the collected, suspended biofilm was mixed with 5 μl of the LDH Assay Lysis Solution in a 96-well microtiter plate, and incubated at 37° C for 45 minutes. 100 μl of LDH Assay mixture was added to the cell

lysate (LDH Assay Cofactor Preparation: LDH Assay Substrate: LDH Dye Solution = 1:1:1). The mixture was kept in the dark and incubated at room temperature for 30 minutes. To terminate the reaction, 50 μ l of Stop Solution was added to the mixture. We measured the absorbance of the samples at OD_{490nm} and the background absorbance at OD_{690nm}. The background absorbance values were subtracted from the samples readings.

- EPS amount: Phenol-Sulfuric Acid Colometric Assay

The amount of EPS was determined using a previously described protocol. (5) Briefly, 50 μ l of the biofilm of each sample was transferred to a 96-well microtiter plate. For each sample, 150 μ l of concentrated sulfuric acid was added. Immediately after that, 30 μ l of a 5% phenol solution was added to the mixture and heated to 90° C for 5 minutes. After cooling the plate at room temperature for 5 minutes the absorbance was measured at OD_{750nm}. (5)

All LDH and EPS values were normalized using the protein concentration of each biofilm sample, which was obtained using the RC DC Protein Assay (Bio-Rad Laboratories, Inc. Hercules, CA, USA) following the manufacturer's instructions.

- Bacterial Viability

At the end of each time point, biofilm samples from all groups were serially diluted to 1:10³, 1:10⁴, and 1:10⁶ (using 0.9% sterile saline). Samples were plated on Blood Agar Plates (Thermo Scientific™, Remel, Lenexa, KS, USA) using a Spread Plate Procedure. ⁶⁹ To determine bacterial counts, mean log₁₀ CFU/ml values were calculated.

Statistical Analysis

All test results in both aims were analyzed using two-way ANOVA. For the first aim, factors for exposure period (i.e. T1, T2, and T3) and treatment type (NaF, SnF₂, AmF, and DIW) as well as the interactions between them were analyzed. For the second aim, all test results from the T1 exposure period collected at different time-points were analyzed with factors for collection time (immediate collection, 3-day post-treatment, and 6-day post-treatment) and treatment type as well as the interactions between them. Similarly, all test results from group T2 collected at different time-points were analyzed with factors for collection time (immediate collection, 4-day post-treatment) and treatment type as well as the interactions between them.

In all above two-way ANOVA models, the experimental unit was included as a random effect. All pair-wise comparisons from ANOVA analysis were made using Fisher's Protected Least Significant Differences to control the overall significance level at 5%. VHN_{change}, ΔZ , L, log₁₀ CFU/ml, EPS amount and LDH activity were summarized by exposure period, collection time and treatment type.

5.3. Results

Aim 1: To explore the influence of biofilm's maturation and time of exposure to fluoride treatments on fluoride's anticaries efficacy

Specimen images 2 and 3 show biofilm-coated enamel surfaces on the day of collection for different time points, treated with 4 different treatment types. In both parts of this aim, the ANOVA analyses tested the exposure period (T1, T2, and T3) and treatment type, as well as the interaction between them. Looking at carious lesion's

severity, the results from the immediate collection of the biofilm and enamel samples demonstrated that the change in surface microhardness was influenced by exposure period: early exposure (T1) to treatment resulted in significantly lower VHN_{change} when compared to later exposure (T2 and T3). Exposure period also influenced the ΔZ and L values in all treatment types (Table 5.2a).

Considering biofilm cariogenicity (Table 5.2b), the results from the immediate collection of the biofilm demonstrated a significant interaction between the two variables only in LDH activity and viability, but not for EPS amount. The more mature biofilm had higher viability when compared to early biofilm, even when the exposure to treatment was very close to collection time (T3). SnF_2 and AmF always showed a higher effect on bacterial viability when compared to NaF and DIW. Earlier exposure to treatment (T1 and T2) resulted in significant reduction in LDH activity than late exposure (T3). The EPS amount data showed significant effect of exposure period and treatment type ($p < 0.0001$) but not the interaction between the two variables ($p = 0.1270$). Both SnF_2 and AmF resulted in lower EPS amount when compared to NaF and DIW.

The second part of this aim is the ANOVA analysis of 10-day old biofilms and enamel specimens exposed to treatments at different maturation stages (i.e. exposure periods) (Figure 5.1). The carious lesion severity was affected by the variables tested in this study. Samples in the “early exposure” groups (i.e. T1) showed a protective effect of fluoride when compared to control (Table 5.3a). Early exposure (T1) resulted in producing a protective effect in VHN_{change} in all fluoride-treated groups, while later exposure (T2 & T3) prevented the progress of the already-formed lesion only when treated with SnF_2 and AmF. The L data also showed a protective effect in earlier

exposure (T1 and T2) of the biofilm to treatments when compared to late exposure (T3). ΔZ was significantly affected as well by exposure period (Table 5.3a).

When testing biofilm cariogenicity, the two-way ANOVA showed a significant effect of both variables. The interaction between these two variables was significant in bacterial viability and EPS amount. However, the LDH analysis showed a statistical significance in the treatment type only.

Regarding biofilm's viability, SnF₂ and AmF were always superior in their effect when compared to NaF and DIW. Exposure period (T1, T2, and T3) was an influencing factor in biofilm's viability except when treated with SnF₂ (Table 5.3b). The LDH activity was inhibited in the SnF₂ and AmF groups (when compared to NaF and DIW) regardless of the exposure period. The biofilm showed an increased tolerance to NaF, in terms of lactic acid production, in all time periods. On the other hand, the EPS amount showed a significant interaction between both variables ($p < 0.0001$). Looking at exposure periods, late exposure to treatment still showed more presence of EPS within the 10-day old biofilm when compared to earlier exposure (T1 and T2). Also, SnF₂ and AmF showed a significant effect when compared to NaF and DIW in all exposure periods (Table 5.3b).

Aim 2: To explore the recovery of biofilm's cariogenicity after exposure to fluoride treatments using two exposure periods

Specimen images 4 and 5 show biofilm-coated enamel surfaces on the day of collection for different recovery periods, treated with 4 different treatment types. In the first part of this aim, we used early exposure to treatment (T1). We measured and compared lesion severity and biofilm cariogenicity at three time points: immediately after treatment; 3-day post treatment; 6-day post treatment.

The carious lesion severity data showed that VHN_{change} was influenced by treatment type with immediate collection. The further the lesion, the less significant VHN_{change} observed (Table 5.4a). Fluoride type did not create a significant difference in ΔZ and L data in any collection timepoint.

SnF_2 and AmF produced a lasting anticaries effect (LDH and EPS data) even up to 6 days after exposure, while NaF-treated biofilm started to produce higher EPS amounts 3 days post treatment and was able to fully recover (i.e. LDH and EPS data) after 6 days (Table 5.4b).

In the second part of this aim, we used later exposure to treatments (T2). We used two time points for comparison: immediate collection and 4 days post-treatment. The carious lesion severity (ΔZ and L data) was not significantly different between samples with immediate collection and after 4 days recovery in the three treatment tested. However, a significant difference was observed between treatments and controls in both ΔZ and L data (Table 5.5a).

The only treatment that was able to continue affecting biofilm's viability over time is SnF_2 (Table 5.5b). Moreover, the biofilm was only able to recover its cariogenicity (LDH and EPS data) in NaF-treated groups.

5.5. Discussion

Two main aims were explored in this study. First, we evaluated how the timing of exposure to fluoride treatments and its interaction with biofilm maturation can alter fluoride's anticaries activity. Second, we tested the ability of the biofilm to recover after early (T1) and later (T2) exposure periods to fluoride treatments. To our knowledge, this

is the first study testing the interaction between biofilm maturation and different exposure periods to treatments. It is also the first study testing long-term biofilm recovery after treatment with different fluoride compounds.

Our study focused on 3 exposure periods of the biofilm to fluorides: early exposure (T1), later exposure (T2); and late exposure (T3). In each exposure period we tested how fluoride's activity was altered. We also tested whether or not the biofilm was able to recover (in terms of cariogenicity, as well). Based on the results from this study, both biofilm cariogenicity and its recovery are altered not only based on the exposure period, but also according to the fluoride compound used.

Fluoride is the agent of choice preventing the initiation and controlling progression of dental caries.⁹³ Several fluoride compounds are currently being utilized in oral hygiene products, including the studied NaF, AmF and SnF₂. The antibacterial effect of these fluoride compounds does not only come from the anion (F⁻), but also from the cations (Sn⁺² and NH₂⁺).^{94 95 23 78}. This may explain the different outcomes observed on the biofilm cariogenicity and lesion severity when treated with different fluoride compounds.

In the first aim of this study, we tested the variables under two different conditions. We collected the biofilm, in the first condition, after the last day of treatment, regardless of the biofilm's age. In the second condition, we compared 10-day old biofilms that were exposed to treatments at different exposure periods. The carious lesion severity was influenced by both factors: treatment type and exposure period. The results from both, immediate collection (Table 5.2a) and 10-day old biofilm (Table 5.3a) show less variability between treatment types with early exposure. However, when the

exposure to treatment is late, specifically T3, SnF₂ and AmF were more effective when compared to NaF (Tables 5.2a and 5.3a).

When testing biofilm cariogenicity (LDH activity and EPS amount), SnF₂ and AmF had always produced a superior effect when compared to NaF and DIW. With immediate collection of the biofilm (Table 5.2b), SnF₂ and AmF were always able to inhibit the LDH activity. NaF, in the other hand lost its effectiveness when introduced to well-established biofilm at late time (T3) (Table 5.2b). In the second condition, similar findings between the three exposure periods (T1, T2, and T3) were observed in SnF₂ and AmF-treated groups, while NaF failed to inhibit LDH activity nor EPS formation in any group (Table 5.3b).

The amount of EPS was also influenced by exposure period and treatment type. With immediate collection, the later the exposure of the biofilm to treatment, the more EPS was formed (Table 5.2b). In fully mature biofilm (Table 5.3b), late exposure (T3) allowed more tolerance against fluoride compounds, even in SnF₂ and AmF (when compared to T1, T2).

An interesting observation was that the NaF-treated groups, T2 produced the highest LDH and EPS activities. This may be explained as follows: First, early exposure (T1) to NaF may have produced a slight, sustainable inhibitory effect against LDH and EPS activity. Second, the (T3) NaF-treated group was collected immediately after the last day of treatment, allowing a short-term effect of NaF (Table 5.3b). This observation (from T2) was consistent with Dang et al. ⁶ (2016) (given the differences in the two study designs). Dang et al. (2016) concluded in their study that regardless of the NaF concentration used to inhibit biofilm's acidogenicity, the biofilm is fully recoverable.

The second aim tested the biofilm recovery after exposure to treatments. Findings from surface microhardness also showed a protective effect of SnF₂ and AmF after early exposure. The difference in the ΔZ and L values between immediate collection and 6 days of recovery were very slight (all treatment types), suggesting that early exposure may produce a long-term protective effect against more demineralization, regardless of treatment type (Table 5.4a).

All three fluoride compounds when introduced at a later stage (T2) were able to produce a slight reduction in ΔZ and L values (although non-significant) 4 days post-treatment (Table 5.5a). A possible explanation is that the biofilm stored amounts of fluoride that were released over time post-treatment and allowed for some remineralization.

Whether it was an early (T1) or later (T2) exposure to treatment, both SnF₂ and AmF resulted in a sustained antibacterial effect up to 6 days post-treatment (T1) or 4 days post-treatment (T2). Naumova et al. concluded in a recent study that AmF toothpaste has a superior long-term fluoride-bioavailability effect when compared to NaF. ⁹⁶ NaF and DIW-treated groups showed an increase in the activity after treatment; the more time post-treatment, the higher the biofilm activity as well. (Tables 5.4b and 5.5b).

In all parts of this study, we found that irrespective of exposure period or biofilm collection time bacterial viability was not affected. This is similar to what was found in several studies that studied fluoride and virulence factors of the biofilm, ^{6, 7, 19} and recommended in the end focusing on inhibiting biofilm cariogenicity rather than achieving a bactericidal effect.

Our previous study tested biofilm maturation when the biofilm was exposed to fluorides on a daily basis as part of the pH cycling protocol (2 fluoride treatments/day). The pH cycling periods (and hence the number of days the biofilm was exposed to treatment) extended up to 12 days. In this study, we used a different approach, we kept the number of days of exposure to fluorides to 4 days, and consequently, modified the exposure period to early (T1), later (T2), and late (T3) exposure periods. We found that the behavior of the biofilm changes depending on the fluoride compound and exposure period, and not necessarily the number of days of exposure to treatment.

The biofilm model and pH cycling model we used in this study were based on several previous pilot experiments we conducted (data not shown). During these pilot experiments, we incorporated the microbial component as part of the pH cycling model to achieve more clinical relevance. The existing well-established models do not often incorporate a microbial component^{20, 70}; this suggests an increased demand for further studies exploring microbial caries models. Pooled saliva from three donors was used as the source of the salivary bacterial mix. It was reported previously in the literature that collecting different saliva samples from the same donor at different times, and also involving sucrose over time in growing the biofilm, they all lead at the end to overcoming any differences between samples through the increased dominance of certain strains (mainly cariogenic) over time.^{41, 50-52}

We used in our model bovine enamel specimens to grow the biofilm. Lippert and Lynch⁴⁶ (2014) indicated that bovine enamel samples have the exact characteristics of human enamel except faster progressing lesions in bovine enamel. This is not confounding to the use of bovine enamel, especially considering that bovine enamel is

cost-effective and more available than human enamel specimens.⁴⁶ We used an “active attachment” concept, to ensure the bacteria is actively attached to the enamel surface; this prevents the accumulation of layers of bacteria over the sample if it was placed on the base of the well-plate, which results in less clinical relevance.²³ The fluoride concentrations used in our study were 287.5 ppm F. This concentration has significance as it corresponds to the dilution of 1150 ppm fluoride found in toothpastes during toothbrushing (1:3 dilution ratio). Fluoride, when used as toothpaste or mouthwash, is expected to be diluted during expectoration and swallowing.^{17, 62} Finally, we used a pH cycling model, where we initially modified a previously published model³², then optimized the conditions used in the study through pilot experiments measuring pH levels, pH cycling periods, growth medium conditions (mineral saturation).

In several studies,^{7, 19, 30} Pandit et al. (2015) suggested a brief treatment (i.e. 1 minute) of the biofilm with fluoride to control virulence factors such as acidogenicity, acidity and EPS formation to be a potential approach for preventing dental caries. However, Dang et al.⁶ (2016) showed a full recovery of the biofilm post-treatment. Pandit et al.⁷ (2015) showed that fluoride’s effect is concentration dependent up to 100 ppm, then reaching a plateau afterwards. These studies focused on a single species biofilm. They monitored biofilm for relatively short periods (compared to our study). Since increasing the concentration is not expected to produce a stronger anti-virulence effect,⁷ we instead used the concentration that corresponds to over-the-counter oral hygiene products. We chose to test several fluoride compounds on a microcosm biofilm, and longer treatment times during the day (5 minutes; twice/day). In our experiment, we extended the number of “treatment days” to 4 days at each exposure period. Then we

allowed up to 6 days post-treatment recovery. NaF did allow a full recovery of the biofilm's cariogenicity, and this is consistent with the literature that reported how NaF lacks substantivity.^{85, 86}

In this study, some subgroups failed to significantly recover from fluoride treatment. We still do not fully understand the mechanism of biofilm recovery, but further research is needed in this area to also evaluate the influence of the other minerals (i.e. stannous and amine) on inhibiting biofilm's recovery.

Regarding carious lesion severity, ΔZ and L data in particular, this study showed in general that with early exposure to fluorides, less progression of the lesion is observed. This suggests that biofilm may have stored some amounts of fluoride and served as reservoir over time. When we compare this study to our previous study, we may be able to state that whether the fluoride treatment is continuous as part of the daily pH cycling, or introduced at earlier periods of pH cycling, a protective effect of fluorides against demineralization is still observed.

5.6. Conclusion

The results from this study suggest that the earlier exposure of the biofilm to fluoride treatment, the less tolerant the biofilm may become over time, and this also depends on the fluoride compound used. Early treatment may suggest as well the sustainable release of fluoride treatment that was stored within the biofilm and allowed the enamel surface to be protected from further demineralization, but more research is needed in this area. Treating well-established biofilm with SnF₂ and AmF may achieve higher anticaries results when compared to NaF. Bacterial biofilm has the potential for

full cariogenic recovery; to achieve a long-term antibacterial effect in oral hygiene products, SnF₂ and AmF may be the choice in this case.

Future studies may include studying the fluoride concentration within the biofilm and monitor its release in the environment (culture media) and at the biofilm-enamel interface (fluoride retention within the enamel). Another future approach in biofilm maturation studies is focusing on microbial composition and phenotypic changes- we focused our project on biofilm's cariogenic function. A third area of future research is studying the effect of fluoride on EPS architecture, since the variability in EPS architecture across the surface may modify the function of the EPS as a diffusion barrier.

Exposure Period	pH Cycling Days									
	1	2	3	4	5	6	7	8	9	10
T1 (Early Exposure)	pH Cycling With Treatments ←────────────────────────────────→				pH Cycling with DIW ←────────────────────────────────→					
T2 (Later Exposure)	pH Cycling with DIW ←────────→	pH Cycling With Treatments ←────────────────────────────────→				pH Cycling with DIW ←────────────────────────────────→				
T3 (Late Exposure)	pH Cycling with DIW ←────────────────────────────────→						pH Cycling With Treatments ←────────────────────────────────→			

Figure 5.1. pH cycling days. Highlighted days: pH cycling using fluoride treatments; non-highlighted days: pH cycling with DIW instead of fluoride treatments

Table 5.1.a. Group distributions -influence of time of exposure and biofilm maturation on fluoride activity

Exposure Period	Days of Exposure to Fluorides (treatment groups)	Days of Biofilm Collection
T1 (early exposure)	1-4	<ul style="list-style-type: none"> • Part 1- Immediate Collection: day 4 • Part 2- 10-day old biofilm
T2 (later exposure)	3-6	<ul style="list-style-type: none"> • Part 1- Immediate Collection: day 6 • Part 2- 10-day old biofilm
T3 (late exposure)	7-10	<ul style="list-style-type: none"> • Part 1- Immediate Collection: day 10 • Part 2- 10-day old biofilm

Table 5.1.b. Group distributions -biofilm recovery after early (T1) and later (T2) exposure to fluoride treatments

Exposure Period	Days of Exposure to Fluorides (treatment groups)	Days of Biofilm Collection
T1 (early exposure)	1-4	<ul style="list-style-type: none"> • Immediate Collection: Day 4 • 3-day Post-treatment: Day 7 • 6-day Post-treatment: Day 10
T2 (later exposure)	3-6	<ul style="list-style-type: none"> • Immediate Collection: Day 6 • 4-day Post-treatment: Day 10

Table 5.2.a. The influence of biofilm maturation and exposure period to fluoride treatments on lesion severity-Immediate collection. All data are presented as [mean (standard deviation)] as a function of maturation stage and treatment

	Carious Lesion's Severity Mean (SD)																	
	VHN _{change}						L (μm)						ΔZ (%volmin×μm)					
	T1			T2			T3			T1			T2			T3		
NaF	81.7 (6.8)	a,1	89.9 (4.3)	b,1,2	93.4 (2.2)	b,1	47.6 (18.3)	a,1	77 (16.6)	b,1	106.1 (15.6)	c,1	1130 (423)	a,1	2125 (418)	b,1	3114 (525)	c,1
SnF ₂	69.5 (10)	a,2	86.6 (5.4)	b,a	89.1 (4.1)	b,2	50.7 (17.6)	a,1	76.1 (21.6)	b,1	99.7 (31.9)	c,1	1000 (310)	a,1	1876 (569)	b,1	2634 (828)	c,2
AmF	77.2 (7.9)	a,3	88.6 (4.3)	b,1,2	90.8 (4.1)	b,1,2	53.9 (36.2)	a,1	78.9 (18.5)	b,1	97.4 (12.7)	c,1	1332 (880)	a,1	2161 (513)	b,1	2682 (504)	c,2
DIW	89 (5.8)	4	91.8 (4.5)	2	91.4 (3.8)	1,2	77.5 (15.5)	a,2	92.2 (18.1)	b,2	109.6 (27.9)	c,2	2201 (653)	a,2	2662 (640)	b,2	2947 (1142)	b,1,2

Letters: significance between exposure periods

Numbers: significance between treatment types

Table 5.2.b. The influence of biofilm maturation and exposure period to fluoride treatments on biofilm cariogenicity-Immediate collection. All data are presented as [mean (standard deviation)] as a function of maturation stage and treatment

	Biofilm Cariogenicity Mean (SD)																									
	LDH activity (µg/ml)						EPS amount (µg/ml)						Viability (Log ₁₀ CFU/ml)													
	T1			T2			T3			T1			T2			T3										
NaF	1.7 (1.4)	a,1		4.3 (2.4)	b,1		8.6 (4.1)	c,1		3.5 (1.4)	a,1		9.7 (14.3)	b,1		16.5 (7.7)	c,1		7.5 (0.6)	a,1		8.1 (0.6)	b,1		7.9 (0.5)	b,1
SnF ₂	0.3 (0.1)	a,2		0.6 (0.2)	a,2		1.7 (1)	b,2		2.8 (1.4)	a,2		3.2 (1.2)	b,2		11.9 (6.5)	c,2		6.4 (0.6)	a,2		7.1 (0.8)	b,2		7.1 (0.5)	b,2
AmF	0.2 (0.4)	a,2		0.7 (0.5)	a,2		2 (1.4)	b,2		2.5 (1)	a,2		3.2 (0.7)	b,2		12.4 (7.1)	c,2		5.6 (0.5)	a,3		6.2 (0.9)	b,3		3.9 (0.5)	b,3
DIW	1.7 (1.2)	a,1		3.7 (2)	b,1		10.8 (4.2)	c,3		4 (2.2)	a,1		7.5 (2.5)	b,1		19.7 (10.4)	c,1		7.7 (0.4)	a,1		8.3 (0.3)	b,1		8.5 (0.3)	c,4
Letters: significance between exposure periods Numbers: significance between treatment types																										

Table 5.3.a. The influence of biofilm maturation and exposure period to fluoride treatments on lesion severity-10-day old biofilm. All data are presented as [mean (standard deviation)] as a function of maturation stage and treatment

Carious Lesion's Severity Mean (SD)																										
VHN _{change}						L (μm)						ΔZ (%volmin×μm)														
T1			T2			T3			T1			T2			T3			T1			T2			T3		
NaF	83.1 (6.1)	a,1	90.1 (3.5)	b,1,2	93.4 (2.2)	c,1	60.5 (18.8)	a,1	74.2 (18)	a,1	106.1 (15.6)	b	1498 (449)	a,1	1931 (461)	b,1	3114 (525)	c,1								
SnF ₂	78.2 (7.2)	a,2	87.9 (3.5)	b,1	89.1 (4.1)	b,2	62.4 (23.8)	a,1	72.6 (20.1)	a,1	99.7 (31.9)	b	1357 (416)	a,1	1820 (452)	b,1	2634 (828)	c,2								
AmF	82.6 (4.8)	a,1	89.4 (3.4)	b,1,2	90.8 (4.1)	b,1,2	66.4 (26.4)	a,1	78.4 (14.6)	a,1	97.4 (12.7)	b	1584 (572)	a,1	2052 (365)	b,1	2682 (504)	c,2								
DIW	92.1 (3.4)	3	91.5 (4.3)	2	91.4 (3.8)	1,2	124.1 (18.4)	a,2	118.4 (23)	a,b,2	109.6 (27.9)	b	3551 (773)	a,2	3487 (838)	a,2	2947 (1142)	b,1,2								
Letters: significance between exposure periods Numbers: significance between treatment types																										

Table 5.3.b. The influence of biofilm maturation and exposure period to fluoride treatments on biofilm cariogenicity-10-day old biofilm. All data are presented as [mean (standard deviation)] as a function of maturation stage and treatment

	Biofilm Cariogenicity Mean (SD)																	
	LDH activity (µg/ml)						EPS amount (µg/ml)						Viability (Log ₁₀ CFU/ml)					
	T1		T2		T3		T1		T2		T3		T1		T2		T3	
NaF	8.6 (4.7)	1	10.3 (5.4)	1	8.6 (4.1)	1	14.9 (9.6)	a,1	20.1 (9.2)	b,1	16.5 (7.7)	a,b,1	8.1 (0.6)	a,b,1	8.5 (0.1)	a,1	7.9 (0.5)	b,1
SnF ₂	1.4 (1.5)	2	1.2 (1.1)	2	1.7 (1)	2	3.2 (2.1)	a,2	4 (2.2)	a,2	11.9 (6.5)	b,2	7.1 (0.8)	2	7.1 (0.8)	2	7.1 (0.5)	2
AmF	0.7 (0.8)	2	0.8 (0.5)	2	2 (1.4)	2	2.9 (1.2)	a,2	4 (1.1)	a,2	12.4 (7.1)	b,2	6.2 (0.9)	a,3	6.2 (0.3)	a,3	3.9 (0.5)	b,3
DIW	9.8 (4.3)	3	10.2 (3.4)	3	10.8 (4.2)	3	18.6 (7.4)	1	21.4 (9.1)	1	19.7 (10.4)	1	8.3 (0.3)	1	8.4 (0.3)	1	8.5 (0.3)	4
Letters: significance between exposure periods Numbers: significance between treatment types																		

Table 5.4.a. The influence of biofilm recovery after early exposure to fluoride treatments on carious lesion severity. All data are presented as [mean (standard deviation)] as a function of maturation stage and treatment

	Carious Lesion's Severity Mean (SD)											
	VHN _{change}			L (µm)			ΔZ (%volmin×µm)					
	Immediate Collection	3-days Recovery	6-days Recovery	Immediate Collection	3-days Recovery	6-days Recovery	Immediate Collection	3-days Recovery	6-days Recovery	Immediate Collection	3-days Recovery	6-days Recovery
NaF	81.7 (6.8) 1	80.8 (10.6) 1	83.1 (6.1) 1	47.6 (18.3) 1	54 (12.8) 1	60.5 (18.8) 1	1130 (423) a,1	1354 (325) a,b,1	1499 (449) b,1			
SnF₂	69.5 (10) a,2	72.6 (10.5) a,2	78.2 (7.2) b,1	50.7 (17.6) 1	55.1 (19.6) 1	62.4 (23.8) 1	1000 (310) a,1	1262 (414) a,b,1	1357 (416) b,1			
AmF	77.2 (7.9) a,1	72.2 (14.1) a,2	82.6 (4.8) b,1	53.9 (36.2) a,b,1	45.1 (15.7) a,1	66.4 (26.4) b,1	1332 (880) a,b,1	1089 (378) a,1	1584 (572) b,1			
DIW	89 (5.8) 3	91.8 (3.4) 3	92.1 (3.4) 2	77.5 (15.5) a,2	96.8 (12) b,2	124.1 (18.4) c,2	2201 (653) a,2	2792 (505) b,2	3551 (773) c,2			

Letters: significance between exposure periods
Numbers: significance between treatment types

Table 5.4.b. The influence of early exposure to fluoride treatments on biofilm recovery-biofilm cariogenicity. All data are presented as [mean (standard deviation)] as a function of maturation stage and treatment

Biofilm's Cariogenicity Mean (SD)																		
	LDH activity (µg/ml)					EPS amount (µg/ml)					Viability (Log ₁₀ CFU/ml)							
	Immediate Collection		3-days Recovery		6-days Recovery	Immediate Collection		3-days Recovery		6-days Recovery	Immediate Collection		3-days Recovery		6-days Recovery			
NaF	1.7 (1.4)	a,1	4.6 (2.9)	b,1	8.6 (4.7)	c,1	3.5 (1.4)	a	5.6 (3)	a,1	14.9 (9.6)	a,1	7.5 (0.6)	a,1	7.9 (0.6)	a,b,1	8.1 (0.6)	b,1
SnF ₂	0.3 (0.1)	2	0.5 (0.2)	2	1.4 (1.5)	2	2.8 (1.4)		2.3 (1.5)	2	3.2 (2.1)	2	6.4 (0.6)	a,2	6.8 (0.6)	a,b,2	7.1 (0.8)	b,2
AmF	0.2 (0.4)	2	0.4 (0.3)	2	0.7 (0.8)	2	2.5 (1)		2 (0.6)	2	2.9 (1.2)	2	5.6 (0.5)	a,3	7.1 (0.4)	b,2	6.2 (0.9)	c,3
DIW	1.7 (1.2)	a,1	5.1 (2.8)	b,1	9.8 (4.3)	c,3	4 (2.2)	a	7.6 (2.6)	b,1	18.6 (7.4)	c,3	7.7 (0.4)	a,1	8.7 (0.2)	b,3	8.3 (0.3)	b,1
Letters: significance between exposure periods Numbers: significance between treatment types																		

Table 5.5.a. The influence of biofilm recovery after late exposure to fluoride treatments on carious lesion severity. All data are presented as [mean (standard deviation)] as a function of maturation stage and treatment

Carious Lesion's Severity Mean (SD)												
VHN _{change}				L (μm)				ΔZ (%volmin×μm)				
Immediate Collection		4-days Recovery		Immediate Collection		4-days Recovery		Immediate Collection		4-days Recovery		
NaF	89.9 (4.3)	1,3	90.1 (3.5)	1,3	77 (16.6)	1	74.2 (18)	1	2125 (418)	1	1931 (461)	1
SnF ₂	86.6 (5.4)	2	87.9 (3.5)	2	76.1 (21.6)	1	72.6 (20.1)	1	1876 (569)	1	1820 (452)	1
AmF	88.6 (4.3)	3	89.4 (3.4)	3	78.9 (18.5)	1	78.4 (14.6)	1	2161 (513)	1	2052 (365)	1
DIW	91.8 (4.5)	1	91.5 (4.3)	1	92.2 (18.1)	a,2	118.4 (23)	b,2	2662 (640)	a,2	3487 (838)	b,2

Letters: significance between exposure periods

Numbers: significance between treatment types

Table 5.5.b. The influence of late exposure to fluoride treatments on biofilm recovery-biofilm cariogenicity. All data are presented as [mean (standard deviation)] as a function of maturation stage and treatment

Biofilm's Cariogenicity Mean (SD)																	
LDH activity (µg/ml)				EPS amount (µg/ml)				Viability (Log ₁₀ CFU/ml)									
Immediate Collection			4-days Recovery			Immediate Collection			4-days Recovery			Immediate Collection			4-days Recovery		
NaF	4.3 (2.4)	a,1	10.3 (5.4)	b,1	9.7 (14.3)	a,1	20.1 (9.2)	b,1	8.1 (0.6)	a,1	8.5 (0.1)	b,1					
SnF ₂	0.6 (0.2)	2	1.2 (1.1)	2	3.2 (1.2)	2	4 (2.2)	2	6.7 (0.8)	2	7.1 (0.8)	2					
AmF	0.7 (0.5)	2	0.8 (0.5)	2	3.2 (0.7)	2	4 (1.1)	2	4.4 (0.4)	a,3	6.2 (0.3)	b,3					
DIW	3.7 (2)	a,1	10.2 (3.4)	b,1	7.5 (2.5)	b,1,2	21.4 (9.1)	b,1	8.1 (0.5)	a,1	8.4 (0.3)	b,1					

Letters: significance between exposure periods

Numbers: significance between treatment types

CHAPTER 6: DISCUSSION AND CONCLUSIONS

6.1. Discussion

Dental biofilm maturation involves alterations in its characteristics such as composition and architecture.¹⁴ These changes may lead to an increased number of acidogenic (i.e. cariogenic) bacteria and also the amount of EPS formed. Fluoride has been documented as the gold standard in controlling the disease⁹³; we are interested in determining how fluoride interferes with biofilm's virulence factors. With alterations occurring within the biofilm at each maturation stage, we expect an alteration in its interaction with anticaries agents (i.e. fluorides).¹⁶ Our study addressed gap in previous research, which mostly focused on biofilms at certain age (timepoint, degree of maturation).^{1, 23, 29}. To our knowledge, our research (with all its components) is the first to study how the biofilm at each maturation stage modulates the anticaries effect of fluoride compounds. This is also the first research studying biofilm recovery after early and later exposure to fluoride compounds.

In chapters 2 and 3, we focused on testing several variables as components of a standard, clinically relevant, and reproducible microbial caries model. The reason we wanted to test these variables is due to the lack of a standard microbial caries model. We found that surface conditioning using sterile saliva does not influence the net mineral loss of enamel substrate. We also tested the influence of sucrose concentration within the media on lesion severity. It was found previously that carbohydrate concentration modifies biofilm composition.^{56, 57}; we confirmed, in our study, that the severity of the lesion is concentration-dependent.

The third factor we tested is substrate type. We found that although the actual values of lesion severity (VHN_{change} , ΔZ and L) were higher in bovine samples, the trend in lesion progression across maturation stages was similar. As Lippert and Lynch (2014) reported that although they had similar characteristics, lesions formed over bovine enamel progress at a faster rate when compared to human enamel.⁴⁶

Another challenge we explored in our model development studies is the issue of uneven biofilm thickness across the substrate surface. This problem has been mentioned and explored by Zero⁷³ (1995) in a study that compared lesion characteristics of specimens covered with gauze to non-covered specimens. The application of protective mesh over our samples ended up to be a confounder that influenced LDH activity and amount of EPS. Moreover, the mesh factor was not a significant factor in lesion severity. Therefore, this area of research still needs more exploration to find ways to create a thick, even biofilm layer over the surface.

In chapter 4, we applied the model developed in chapters 2 and 3 to test biofilm maturation and different fluoride compounds. SnF_2 and AmF continued to produce a higher anticaries effect over time, while NaF lost its anticaries efficacy against more mature biofilm. Previous studies reported that NaF lacks sustainable antimicrobial effect.^{77, 86} Although the biofilm data showed altered tolerance levels (i.e. LDH and EPS activities) based on the treatment type and maturation stage, this did not reflect on the lesion severity. The differences in lesion severity between maturation stages were nonsignificant. This suggests that the daily exposure to fluoride may lead to precipitation of fluoride within the biofilm, which may result in slow release into the environment (i.e. biofilm-enamel interface).^{69, 82, 83}

In chapter 5 we used a different approach. While using the same pH cycling model we developed, we evaluated if modifying exposure periods to three fluoride compounds would have an impact on the biofilm cariogenicity itself and the biofilm-induced lesion characteristics. We found that based on the fluoride compound used, the exposure period may be critical. This indicates that the anticaries effect does not only come from the anion (F^-), but also from the corresponding cations, and this is consistent to what was reported previously in the literature.^{23, 78, 94, 95}

Some studies have explored biofilm recovery after brief fluoride treatment. Dang et al.⁶ (2016) showed full recovery of the biofilm post-treatment. Pandit et al.⁷ (2015) showed that the fluoride effect is concentration dependent up to 100 ppm, reaching a plateau afterwards. When we examined biofilm recovery after early and later exposure to fluoride, we found that biofilm recovery depends on treatment type more than exposure period. Our results showed that exposing an enamel surface at a later stage (after it was already demineralized for some time) to SnF_2 and AmF , there is a possibility for slight remineralization, although this was nonsignificant. This is, again, consistent with our suggestion from chapter 4: some amounts of fluoride may have been stored within the biofilm during exposure periods.

As mentioned earlier, the model we developed and tested here was based on a series of studies. We optimized the demineralization and remineralization pH levels, pH cycling periods, and growth media conditions (i.e. mineral saturation and sucrose concentrations). Based on these preliminary data, we chose the conditions used in this study. We used an active attachment model to ensure that the bacterial layers formed over the surface are not just sedimented cells, but rather attached to the enamel surface and to

each other.²³ We used a microcosm biofilm for more clinical relevance. The source of the bacterial biofilm was pooled saliva from three donors. It has been documented previously how growing microcosm biofilms over time in the presence of sucrose can overcome any variability within the biofilm, even if the samples were obtained from different sources or collected from the same donor at different times. We tested our pooled saliva for the presence of *Streptococcus mutans* and *Lactobacilli* using selective agars. The results confirmed the presence of both species. We used a daily pH cycling model in-vitro in our studies in order to mimic daily oral environment. This gives the model its strength of being reproducible.

In chapter 3, the fluoride concentration used was 18.4 mM NaF (772.5 ppm F). Because we were testing other variables in this experiment (i.e. substrate type and protective mesh application), we wanted all other variables to be based on previously tested models; Zhang et al. (2015) used this concentration in their model.³² When we wanted to adopt our model (developed in chapters 2 and 3) to the subsequent studies (chapters 4 and 5), we modified the fluoride concentrations and compounds to correspond to concentrations in toothpaste: we used a concentration of 287.5 ppm F⁻ in all compounds, which mimicked the dilution of 1150 ppm fluoride found in toothpastes during toothbrushing (1:3 dilution ratio).

Our studies focused on biofilm-induced carious lesion characteristics. We studied each maturation stage and how biofilm function (as in being cariogenic) changes at each stage, and how as it matures it modulates the anticaries action of fluoride compounds. Future studies may include focusing on biofilm composition and phenotypic changes at each Maturation stage. Another area of research is testing how surface conditioning may

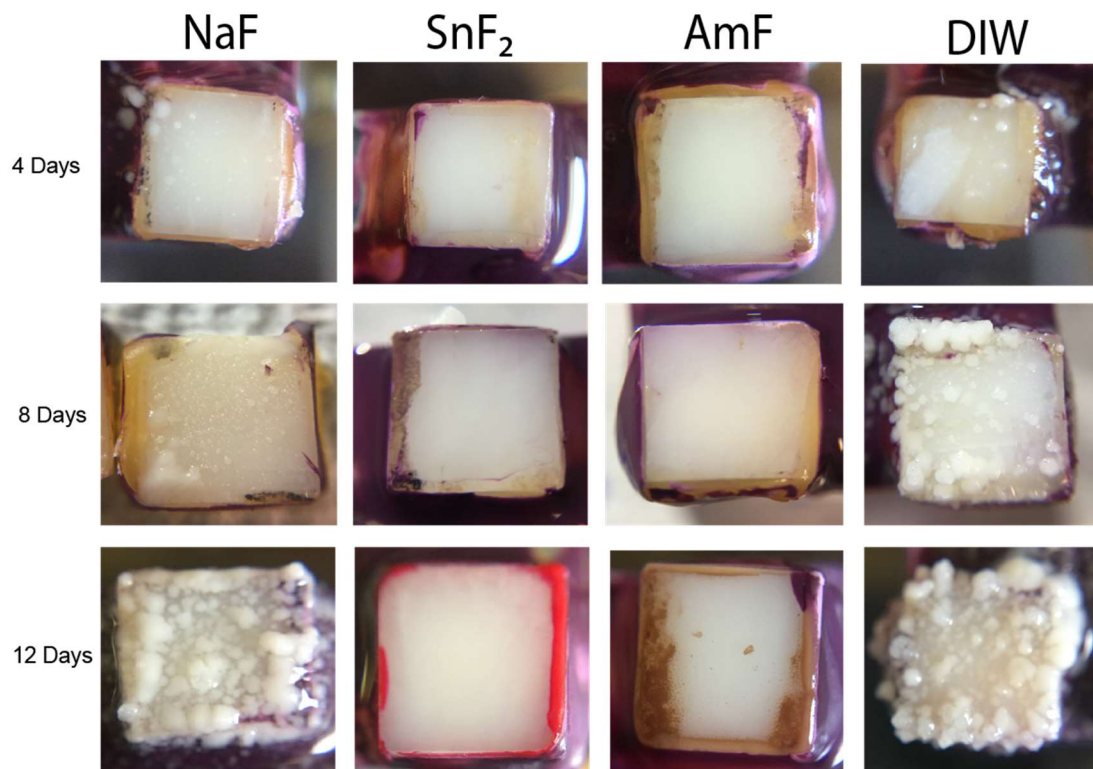
affect biofilm characteristics. Exploring the formation of a uniform biofilm over an enamel surface through modifying surface characteristics is an essential area in microbial caries research. Another area of research may be more interpretation of the results of chapters 4 and 5 through analyzing mineral contents within the biofilm (e.g. fluoride concentrations) for more understanding of the patterns that occurred in lesion progression in both studies.

6.2. Conclusions

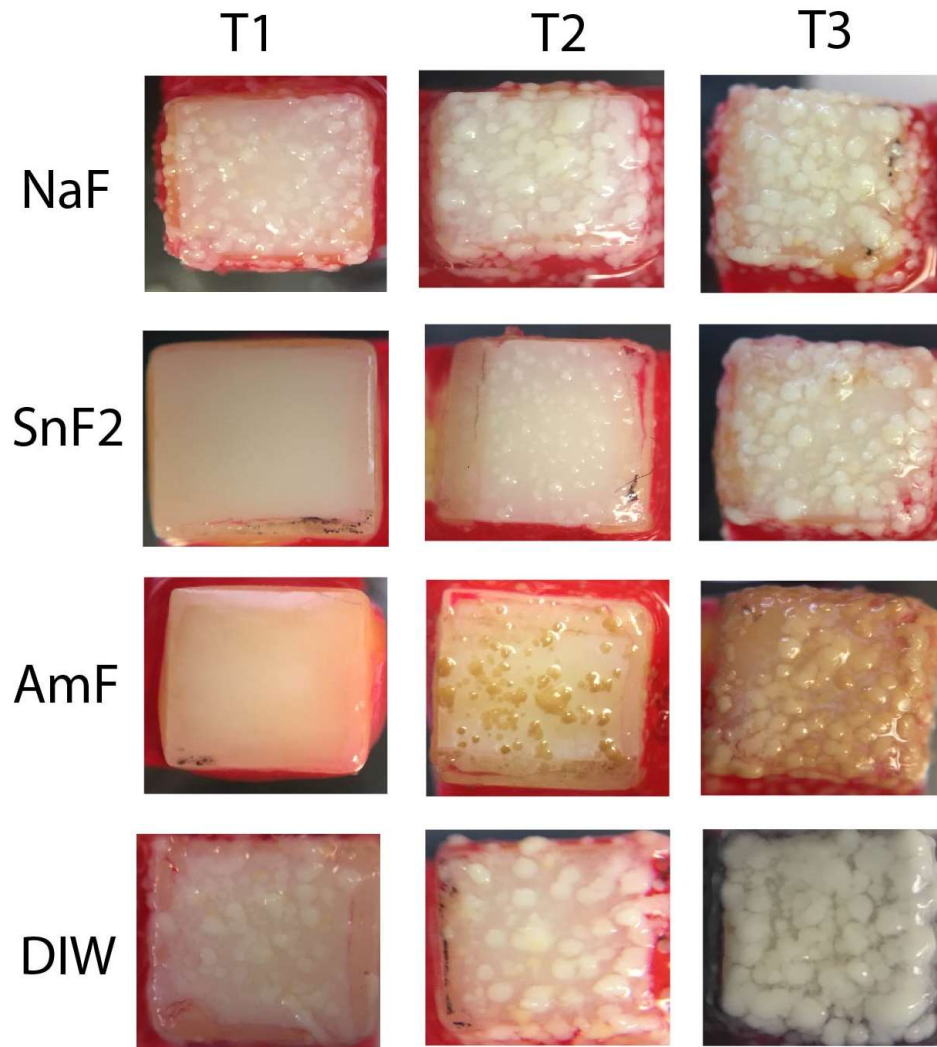
Within the limitations of each study, we conclude from chapter 2 that surface conditioning of the enamel surface does not influence biofilm-mediated enamel caries lesion formation as measured by TMR. In chapter 3, when studying biofilm maturation, both substrate types (human and bovine) are useful in in-vitro microbial caries models. The biofilm-induced lesion in both substrate types progresses similarly. Using a protective mesh may be a confounder to accurately analyze the biofilm formed over the enamel surface.

In chapter 4, we concluded that biofilm tolerance increases as the biofilm matures. Each biofilm maturation stage may influence the selection of fluoride compounds to achieve an optimum cariostatic effect.

In chapter 5, both exposure period and treatment type influence biofilm cariogenicity and biofilm-induced lesion severity. They also influence the ability of the biofilm to recover its cariogenicity. If enamel and biofilm were to be exposed later to fluoride, SnF_2 and AmF may achieve higher anticaries results when compared to NaF .

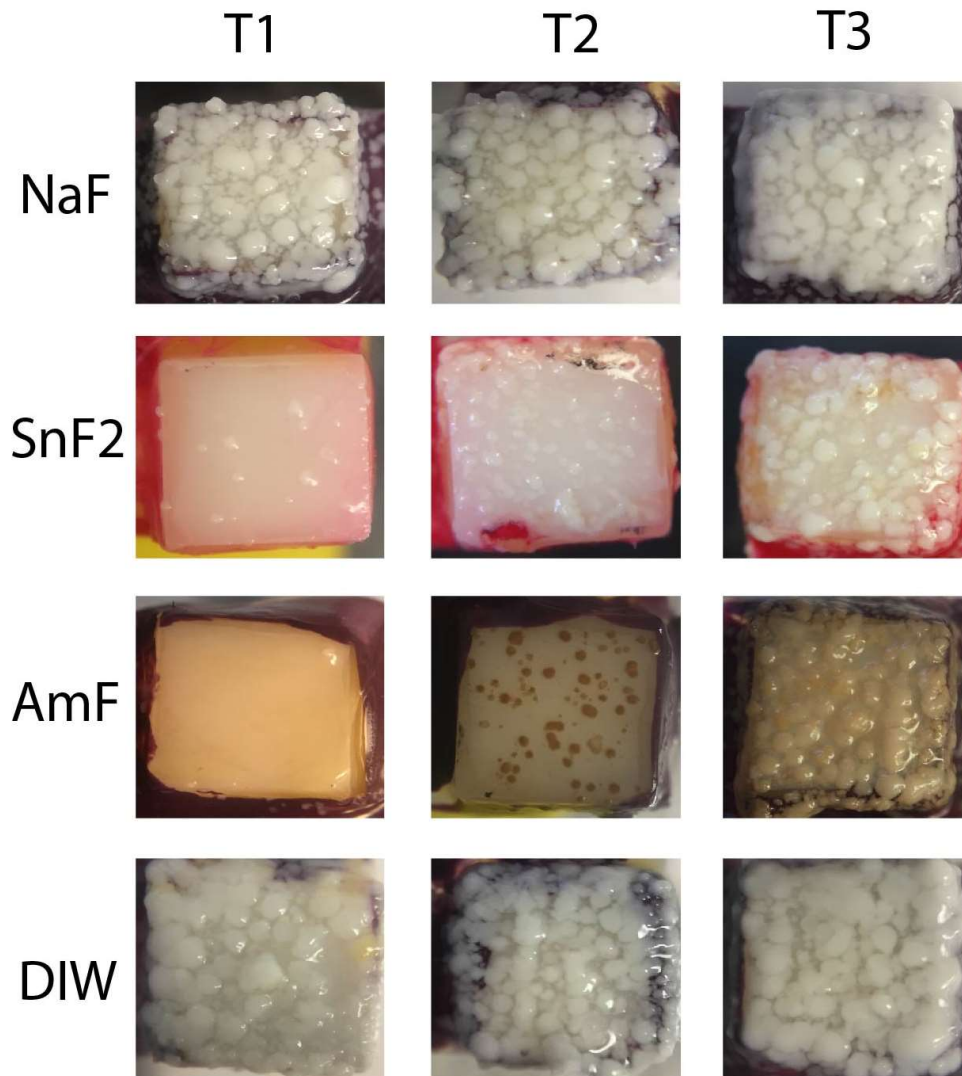


Specimen Image 1. Experiment from chapter 4. Biofilm and enamel specimens after 4, 8, and 12 days of daily pH cycling and treatment with different fluoride compounds

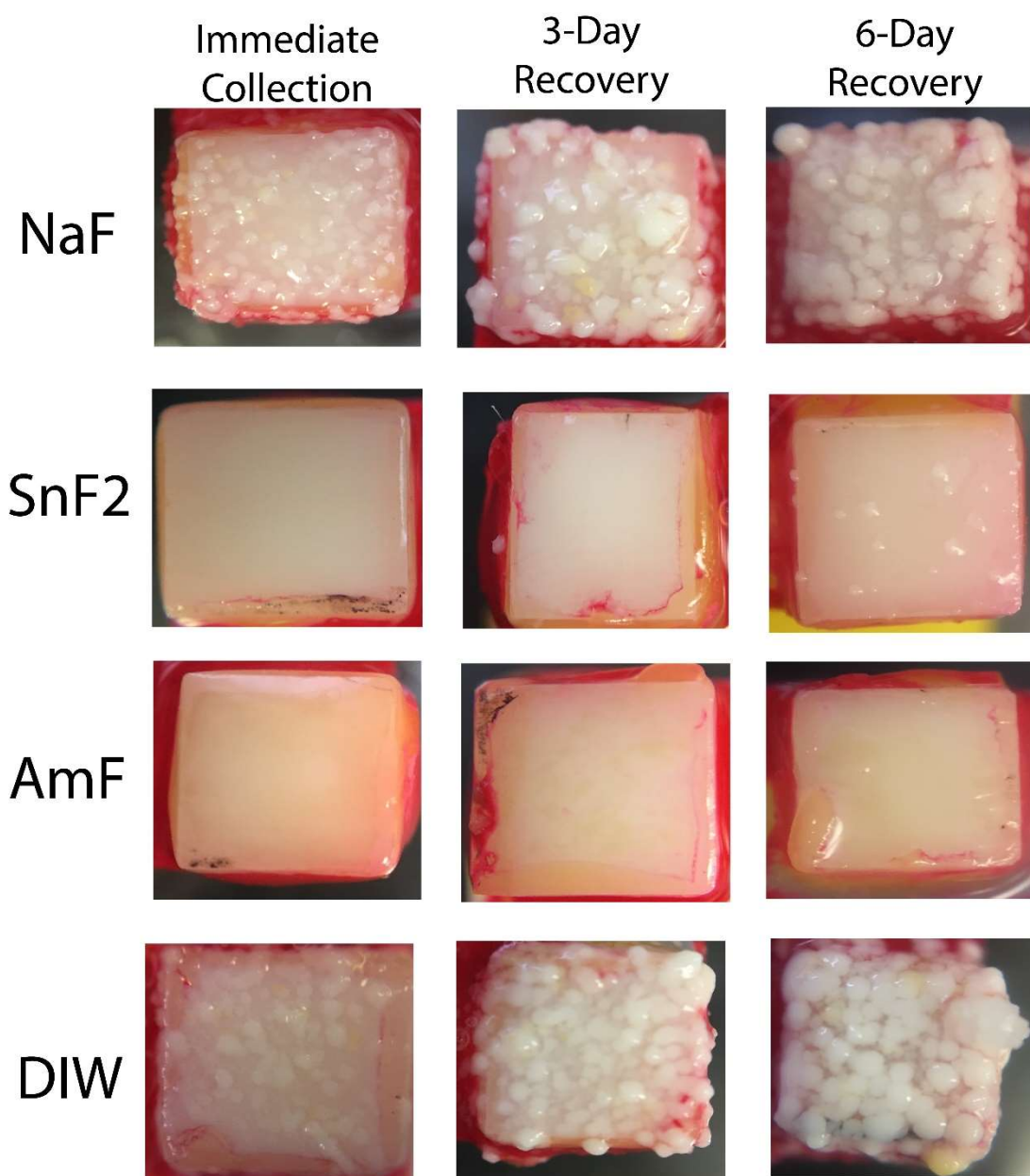


Specimen Image 2. Experiment from chapter 5. Biofilm and enamel specimens at different exposure times (T1; T2; T3) and treated with different fluoride compounds.

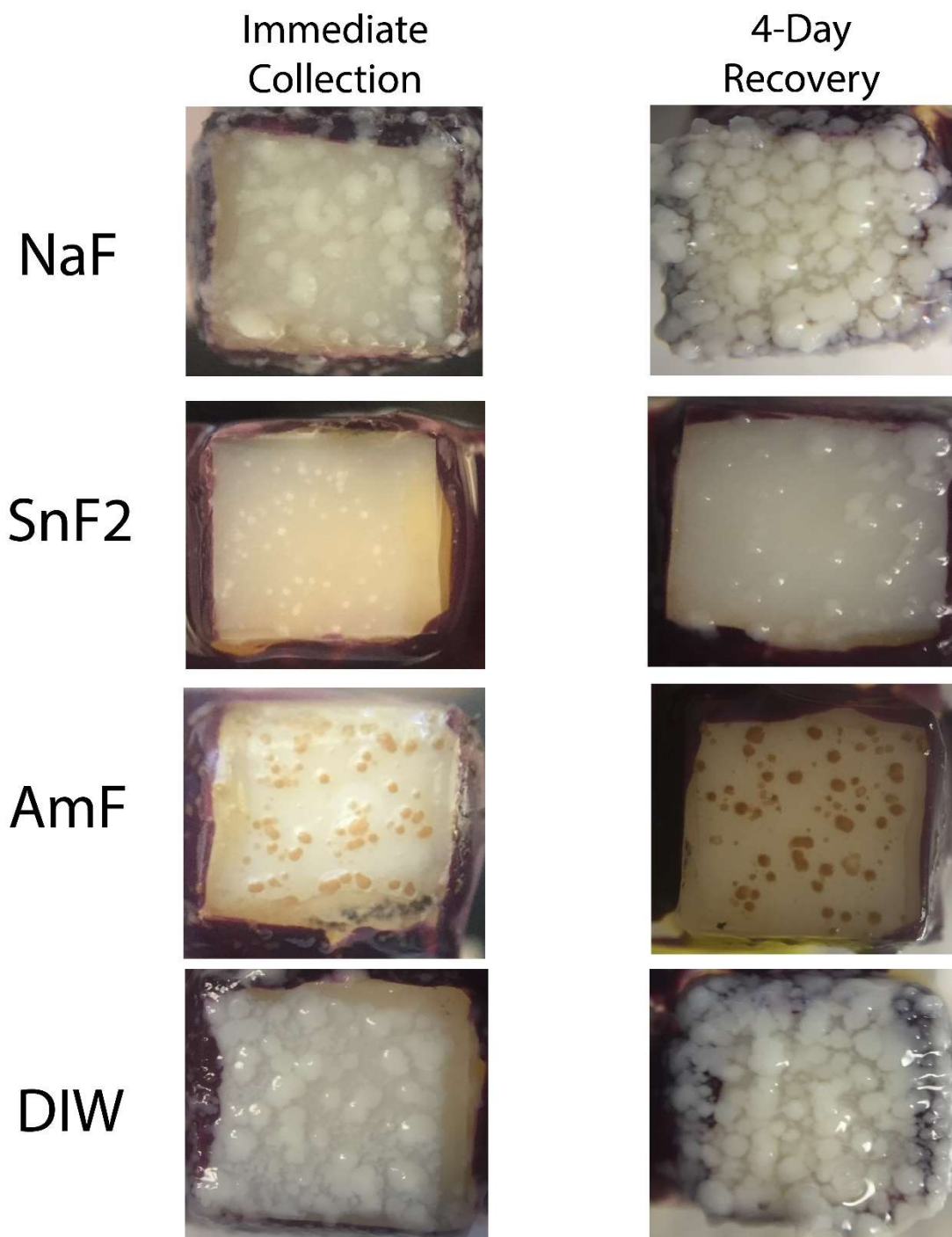
Immediate biofilm collection



Specimen Images 3. Experiment from chapter 5. Biofilm and enamel specimens at different exposure times (T1; T2; T3) and treated with different fluoride compounds. 10-day old biofilm



Specimen Image 4. Experiment from chapter 5. Biofilm recovery. Early exposure (T1) to different fluoride compounds. Three collection timepoints



Specimen Image 5. Experiment from chapter 5. Biofilm recovery. Later exposure (T2) to different fluoride compounds. Two collection timepoints

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Biol. 2019;97:91-6.

CURRICULUM VITAE
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Education Qualifications

May 2006	Bachelor's Degree-Dental Hygiene Dental Health Department, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia
Dec 2012	Preparing Future Faculty Certificate Old Dominion University, Norfolk, VA, USA
May 2013	Master's Degree-Dental Hygiene Old Dominion University, Norfolk, VA, USA
Aug 2018	National Board Dental Hygiene Examination Joint Commission on National Dental Examinations

Professional Experience

2006 - present	Licensed Dental Hygienist Saudi Commission for Health Specialties
2007 - 2014	Teaching Assistant Dental Health Department, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia
2012 - 2013	Adjunct Faculty Gene W. Hirschfeld School of Dental Hygiene, Old Dominion University, Norfolk, VA, USA
2014 - present	Lecturer Dental Health Department, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia

Honors and Awards

2006	Best Intern Dental Hygienist King Khalid University Hospital, Riyadh, Saudi Arabia
2010	Outstanding Teaching Assistant Dental Health Department, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia
2010 - 2019	Full Scholarship The Saudi Arabian Ministry of Education: to pursue graduate education
2013	DENTSPLY/ADHA Graduate Student Clinicians Dental Hygiene Research Program The American Dental Hygienists Association
2013	Gene W. Hirschfeld Excellence in Dental Hygiene Graduate Award Old Dominion University, Norfolk, VA, USA
2014	Nominated for the 2014 United Arab Emirates International Dental Conference & Arab Dental Exhibition Young Research Award Dubai, UAE
2015	Travel Award: Graduate-Professional Educational Grant (G- PEG)-\$500 To attend/present at the IADR/AADR/CADR General Session and Exhibition, Mar 10-15, 2015, Boston, MA

- 2015 **Travel Award: IUPUI Graduate Student Travel Fellowship Award- \$1000**
- To attend/present at the IADR/AADR/CADR General Session and Exhibition, Mar 10-15, 2015, Boston, MA
- 2016 **Travel Award: Graduate-Professional Educational Grant (G-PEG)-\$500**
- To attend the AADR Annual Meeting, Mar 16-19, 2016, Los Angeles, CA
- 2017 **Travel Award: Graduate-Professional Educational Grant (G-PEG)-\$500**
- To attend the 2017 ADEA Annual Session & Exhibition, Mar 18-21, 2017, Long Beach, CA
- 2018 **First Place Winner: poster presentation- “Fluoride’s Anti-Caries Efficacy at Increasing Maturation of a Microcosm Biofilm “**
- The annual meeting for The Indiana Branch of the American Society of Microbiology, Indianapolis, IN
- 2019 **First place winner, poster and oral presentation- “Biofilm Maturation and The Anti-Caries Efficacy of Three Fluoride Compounds”**
- The annual meeting for The Indiana Branch of the American Society of Microbiology, Nashville, IN

Received \$1000 as a scholarship member and had a full vote for the final winning application